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THE EFFECTS OF VIRUSES ON INTRAOCULAR TISSUES.

II. INFECTIONS WITH THE VIRUSES OF HERPES SIMPLEX, FELINE AGRANULOCYTOSIS AND ORNITHOSIS

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In a previous paper[1] it was pointed out that the diversity of cells accessible by direct inoculation in the eye gives this organ particular advantages in the study of the tissue specificity of viruses and certain aspects of the pathogenesis of virus infections. A detailed consideration of infections with the virus of fox encephalitis in the eyes of foxes, dogs, raccoons and cats was presented. In this paper intraocular infections caused by the viruses of herpes simplex, feline agranulocytosis and ornithosis will be described. Each of these viruses produces characteristic inclusion bodies in infected cells. This was an important consideration in choosing them as subjects for investigation inasmuch as the inclusion bodies made possible an accurate recognition of infected cells, and therefore an accurate analysis of the susceptibility or resistance of various kinds of cells in the eye.

Intraocular infection of rabbits with the virus of herpes simplex

Infection of the scarified cornea of the rabbit with the virus of herpes simplex is a well known procedure. A severe keratoconjunctivitis occurs and is characteristically followed by invasion of the nervous system along nerve pathways. Inclusion bodies are found in cells of the corneal and conjunctival epithelium and in neurones and neuroglia.

Goodpasture and Teague[2] showed twenty years ago that in rabbits this virus was capable of infecting many kinds of cells. The characteristic intranuclear inclusion bodies were accepted by them and by subsequent investigators as good evidence of infection of cells in which they occur. Goodpasture and Teague[2] found herpetic inclusion bodies in the following kinds of cells: neurones, neuroglia, ependyma of spinal cord, ganglion cells of retina, tracheal epithelium, hepatic parenchyma, adrenal cortex, interstitial cells of the testis and luteal cells of the ovary. They showed that the virus traversed nerve pathways and that infection of the central nervous system of rabbits followed inoculation of virus into muscle, spleen, testis, adrenal, trachea, skin, liver and ovary. They observed no such infection following injection of the virus subcutaneously or into salivary gland. They also pointed out that Lipschutz[3] had previously described the same sort of inclusions in the nuclei of epithelial cells of the cornea and connective tissue nuclei.

From these earlier reports it was anticipated that intraocular tissues would present an abundance of cells susceptible to the herpes virus and that

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injection of this virus into the eyes of rabbits would cause an extremely severe infection with virtually a wiping out of such susceptible structures. Our expectations were only partially confirmed. Many kinds of intraocular cells proved susceptible, but the severity of the infection was much less than was anticipated.

During the past 6 years a total of 15 rabbits were used in experiments on intraocular infections with the virus of herpes simplex. In spite of the marked susceptibility of many kinds of intraocular cells, the grossly observable manifestations of infection of the eye are not striking. Hyperemia of the iris and of a zone of conjunctiva near the cornea (corona) are the usual changes. There is commonly no conjunctival exudate and little or no corneal haziness. This is true even when the rabbit dies of neural involvement and sections show acute inflammation of most of the visual retina. This slight reaction is in sharp contrast to the severe purulent keratoconjunctivitis that follows inoculation of the scarified cornea with herpes virus.

TABLE 1.—*The distribution of inclusion bodies in the eyes of rabbit C1503.*

Tissue	Herpetic inclusions
Iris	Numerous in fibroblasts, and muscle (radial and circular)
Ciliary body	Fibroblasts, pigmented and nonpigmented epithelium, smooth muscle
Filtration angle	Fibroblasts, macrophages
Miscellaneous	1 endothelial cell of cornea, epithelium of cornea near point of injection

Intracranial injection of mice is a much more sensitive test for herpes virus than intraocular injection of rabbits.

In a titration of a recently isolated strain of herpes virus on January 2, 1943, mice were injected intracranially. A 10^{-3} dilution killed all mice within 10 days; a 10^{-4} dilution killed none in this period of time. Virus was injected into the anterior chamber and into the vitreous body of both eyes of rabbits in the same volume, 0.03 ml, as that injected into the brains of mice. Two rabbits were injected with each dilution of virus. Sections of rabbits killed at 48 and 96 hours failed to show inclusion bodies in any of the eyes injected with a 10^{-2} or 10^{-3} dilution. Therefore, it was concluded that in this experiment readily detectable infection was not produced in the eyes of rabbits by the injection of more than 10 mouse MLD of virus into the anterior chamber and into the vitreous humor. Whether a small area of infection, not readily detectable, was produced in the eyes was not determined, as this experiment was concerned with intraocular infection as a useful means of detecting small amounts of virus.

The distribution of inclusion bodies in the eyes of rabbit C1503 was typical of results seen following injection of virus into the anterior chamber. The findings are shown in table 1.

In these eyes few inclusion bodies were seen back of the ciliary body. Evidence of the susceptibility of cells in this part of the eye is well shown by an experiment made in February, 1946 in which herpes virus of the HF strain was deposited on the retina beneath the superior rectus muscle. The inoculum was 0.1 ml of a 10% suspension of infective mouse brain in Locke's solution and represented between 1500 and 15,000 MLD for mice.

A part of this experiment was a study of the effect of hyaluronidase on intraocular virus infections. Therefore, in some instances virus was mixed with 10% suspension of tissue from the testes of a mature rabbit. This was prepared in Locke's solution and was found to be

highly active in increasing the spread of India ink injected intracutaneously in rabbits.

All animals were killed 4 days after inoculation.

Two sections of each eye in a plane through the area of inoculation and through the optic nerve were later studied, and the distribution of inclusion bodies was mapped. Examples of the results obtained are shown in figure 1.

Extensive herpetic infections of the retina back of the ciliary body were found in all eyes. There was a definite tendency for the infection to spread around the eyes in a zone fairly close to the ciliary body. Spread back over the more central portions of the retina occurred to a variable degree. There was no indication that testicular extract increased the spread of infection. There was a remarkable absence of infection in the ciliary body, iris and other tissues bordering the anterior chamber.

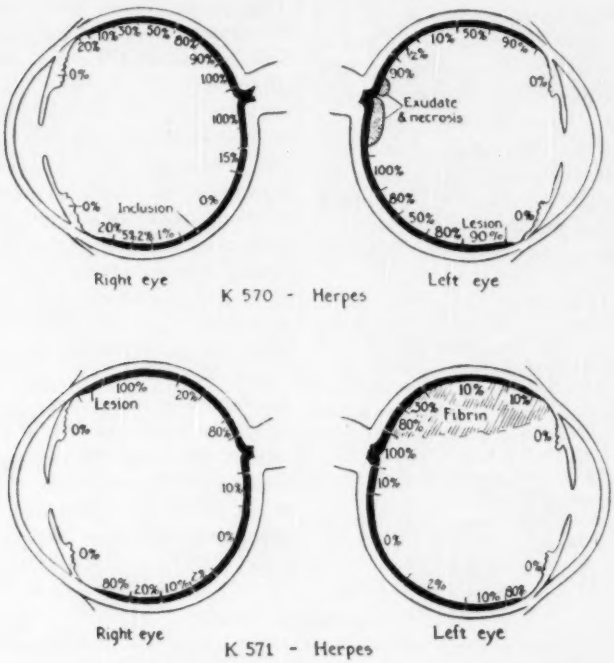

FIG. 1.—Diagrams of distribution of inclusion bodies in retinae of rabbit eyes. Virus was deposited on inner surface of retina. Animals were killed 4 days later. Percentages of ganglion cells and bipolar cells having inclusion bodies are noted. In the region of the ciliary body and iris, data refer to inclusion bodies in the retinal epithelium. "Lesion" refers to the lesion at the site of inoculation.

Inclusion bodies were found in all of the neural elements of the retina. They were in general less readily identified in the darkly stained nuclei of rod and cone cells than in other nuclei. In figure 1 the proportion of nuclei of bipolar cells and ganglion cells having inclusion bodies is recorded.

Infection of the cat eye with the virus of infectious feline agranulocytosis

Syverton and Lawrence and their associates[4,5] and Hammon and Enders[6,7] have studied a disease of cats known as infectious feline agranulocytosis, or malignant panleucopenia of cats. This infection is probably identical with cat distemper[8] and feline enteritis. It is a common disease of cats and is known to be characterized by depression of granulocytes, proliferation of the reticuloendothelial cells of lymph nodes and spleen, and the formation of intranuclear inclusion bodies in cells of the gastrointestinal mucosa, lymph nodes and bronchial mucosa.

In 2 experiments made in 1944, 13 cats were inoculated intraocularly with the virus of feline agranulocytosis. Two cats remained well and were probably immune before injection, and 11 developed the characteristic symptoms of feline agranulocytosis. Six of the cats died of the infection at 5 to 11 days after inoculation, and 3 were killed when moribund on the 6th to 10th day.

Inclusion bodies were sparsely distributed in most of the infected eyes. One animal, K124, that died 5 days after injection of virus, provided exceptionally good material for the study of the kinds of cells infected by the virus. The right eye of K124 had been injected with a 10% suspension of infected cat liver by a route intended to expose as many of the various intraocular tissues as possible.

Small numbers of intranuclear inclusion bodies of the characteristic type were seen in fibroblasts in the iris and ciliary body, and in the pigmented epithelium of the ciliary body. Many inclusions were present in the bipolar cells of the visual retina and in the pigmented epithelium of the visual retina. It is clear that the virus of feline agranulocytosis has the capacity to infect cells of neural origin.

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EXPLANATION OF FIGURES

FIG. 2–9.—Photomicrographs of inclusion bodies in intraocular cells. Arrows point to inclusion bodies.

FIG. 2.—Herpes inclusions in nuclei of bipolar cells in retina of rabbit eye. Hematoxylin and eosin.

FIG. 3.—Feline agranulocytosis inclusion in nucelus of bipolar cell in retina of cat eye. Hematoxylin and eosin.

FIG. 4.—Feline agranulocytosis inclusion in nucleus of bipolar cell in retina of cat eye. Hematoxylin and eosin.

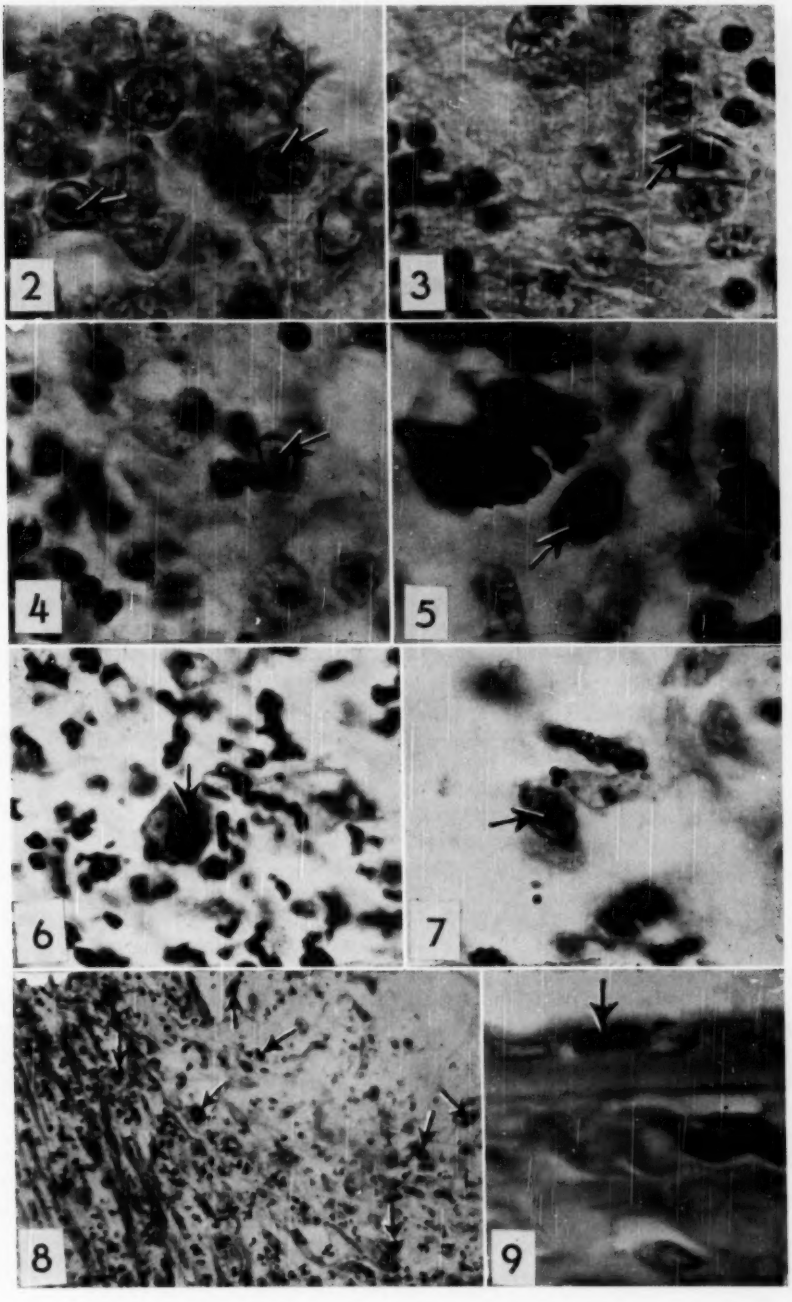
FIG. 5.—Feline agranulocytosis inclusion in nucleus of cell of uncertain type of cell in retina of cat eye. Hematoxylin and eosin.

FIG. 6.—Ornithosis inclusion in cytoplasm of macrophage in anterior chamber of rabbit eye. Giemsa.

FIG. 7.—Feline agranulocytosis inclusion in cell of undetermined type in ciliary body of cat eye. Hematoxylin and eosin.

FIG. 8.—Low power view of filtration angle of eye of rabbit 72 hours after intraocular inoculation of ornithosis virus. Marked cellular infiltration is seen. Distribution of inclusions is shown by arrows. Giemsa.

FIG. 9.—Ornithosis inclusion in cytoplasm of endothelial cell on inner surface of cornea of rabbit eye. Few inclusions were found in cells of this type. Giemsa.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Small numbers of cells with definite inclusion bodies were given presumptive identifications as follows: endothelial cells, pigmented retinal epithelium of the iris, nonpigmented retinal epithelium of the ciliary body, and in one instance, ganglion cell of the retina. If these cells were correctly identified, it is apparent that inclusion bodies occur rarely in cells of these types. They are noted because we feel that these kinds of cells cannot be properly listed as completely resistant to the virus.

Gross inspection of infected eyes showed a remarkable lack of significant evidence of infection. The slight inflammation resulting from trauma quickly subsided, and the eyes did not develop hypermia or other grossly visible evidence of intraocular infection. Retinal examinations were not made.

In discussing infection of the rabbit eye with the virus of herpes simplex, it was noted that many cells of kinds known to be highly susceptible escaped infection. This was even more strikingly true in the case of the virus of feline agranulocytosis in the eyes of cats.

This virus infected fibroblasts and various cells of retinal origin. Yet the total number of infected cells was small, and this fact accounts for the normal appearance of the eyes when viewed grossly.

The inclusion bodies of feline agranulocytosis resemble those of herpes simplex in that they are intranuclear, acidophilic and granular. The fact that the virus of cats is capable of infecting various retinal cells raises a question as to whether this virus, like B virus and pseudorabies, may have some distant but significant relation to the virus of herpes simplex.

Infection of the rabbit eye with ornithosis virus

In a single experiment made in 1943, it was found that a strain of ornithosis virus injected in large dosage into eyes of three rabbits induced a very severe infection with abundant formation of inclusion bodies and grossly evident ocular disturbance.

The virus used in this case was isolated from a severe case of human ornithosis and was received from Dr. F. R. Heilman of the Mayo Clinic. It was maintained in our laboratory by serial intracranial inoculation of mice. An infected mouse brain that had been frozen three weeks was triturated in nine volumes of Ringer's solution and stored 48 hours in a refrigerator. Cultures showed this material to be bacteriologically sterile.

Three rabbits were inoculated in the anterior chamber by means of a needle inserted through the conjunctiva and sclera near the limbus of the cornea, through the ciliary body, behind the iris and through the pupil.

All three rabbits developed a mucopurulent conjunctival exudate at 24 hours and this persisted through the experiment. The animals were killed three, five and seven days after inoculation. All three showed marked thickening (edema) and opacity of the cornea of the inoculated eye. Numerous elementary bodies were seen in smears from the anterior chamber. Sections of the eye from the animal killed at three days showed an acute purulent inflammation of the filtration angle with pus cells and fibrin in the anterior chamber. The iris was notably free of infiltration. Large characteristic cytoplasmic inclusion bodies were found in macrophages in the anterior chamber and adjacent tissues. Similar inclusion bodies were present in cells identified as fibroblasts in the cornea. The endothelial cells lining the inner surface of the cornea were examined with particular interest. These cells were largely

free of infection, but a few showed large well developed inclusion bodies. In any one section of the eye only one or two infected endothelial cells were to be found.

DISCUSSION

There appears to be little doubt that the characteristic inclusion bodies of herpes simplex, feline agranulocytosis and ornithosis occur only in cells infected by the virus. Whether absence of such inclusions is good evidence of complete absence of cellular infection might be questioned. It would appear unlikely that a cell could be the site of growth of any of these viruses to the point of degeneration with release of virus without the formation of the inclusions. An abortive cellular infection that did not reach this stage, is considered to occur in some cells in a number of virus infections and has been commented on notably in the case of poliomyelitis.

In the present study we were chiefly interested in determining which kinds of cells were susceptible to the viruses. To our knowledge there is no evidence to support the idea that cells of a kind refractory to a virus, in the sense that infection never progresses to degeneration of the cells, are susceptible to the extent of undergoing an abortive infection. Therefore, as a working hypothesis, we propose to assume that kinds of cells that never show inclusion bodies are actually resistant to the virus.

A noteworthy finding that has puzzled us is the rare occurrence of inclusion bodies in kinds of cells that in the majority of infected eyes were apparently uninfected. One example is an herpetic inclusion found in one endothelial cell of the cornea of rabbit C1503. In the many eyes infected with herpes virus, no other cell of this type was found to harbor an inclusion. It will be recalled that in the previous paper of this series on intraocular infections with the virus of fox encephalitis, a similar rare occurrence of one inclusion body in each of three cells of normally resistant tissues, a chromatophore, a cell of the nonpigmented epithelium of the ciliary body and a cell of the pigmented epithelium outside the visual retina, was reported.

It is possible that our identification of kinds of cells was in error or that the structures seen were not true inclusion bodies. However, we considered these particular observations of fundamental importance to an understanding of the nature of cellular susceptibility and resistance to viruses, and therefore exercised great care to establish these points accurately. If these observations are correct, a quantitative aspect in susceptibility of tissue cells to viruses is introduced which is difficult to explain. If it is a matter of a rare mutant of the virus having the capacity to infect cells that are normally resistant, one should see on rare occasions a focus of cells infected and possibly an occasional strain of virus exhibiting the capacity to infect these cells extensively. Another hypothetical explanation would be some unusual characteristic of the particular cell, perhaps induced partly by the disturbance of environment caused by infection of nearby tissues.

It is difficult to explain the patchy distribution of inclusion bodies of herpes simplex in the eyes of rabbits, and of feline agranulocytosis in the eyes of cats. Certainly there was no wholesale spread throughout the retina even in the case of herpes virus. This cannot be attributed to insufficient time for spread of virus.

In some areas a scattering of infected cells numbering 2, 10 or 30% of the total was seen; the remaining cells appeared entirely normal. There appears to have been a considerable resistance

to spread of infection from one cell to adjoining cells in some instances.

It can be assumed that spread occurred along nerve pathways, and to some extent outside the nerve pathways, by diffusion from a degenerated cell to an adjoining cell. However, the occurrence of so many normal cells in the presence of a virus with such high pathogenicity for those cells is an interesting, although far from unique, phenomenon. The fundamental question of what limits the spread of viruses from cell to cell does not appear to us to be adequately explained on the basis of antibodies alone.

One wonders whether cellular resistance is enhanced by some nonspecific physiologic alteration stimulated by the presence of infection in neighboring cells. In the experiments of Magrassi[9–12] with herpes virus on what is now called the interference phenomenon, it is difficult to understand how the observed resistance of the entire brain to secondary infection with virulent herpes can be accounted for unless some mechanism other than actual infection with virulent virus was operative.

The mechanism by which the ornithosis virus induced an extremely severe corneal edema is a matter worthy of careful consideration. The marked edema of the cornea seen both grossly and microscopically was in striking contrast to the limited infection of the corneal endothelium. In fox encephalitis one sees a similar corneal edema only in association with extensive infection of corneal endothelium.

It appears that damage to the endothelial membrane as indicated by the severe corneal edema was disproportionately great as compared with the minimal infection of this tissue. This suggests that the toxicity of virus growing abundantly in exudative cells in the anterior chamber may have effected the permeability of this membrane. There is evidence that intravenous injection of rickettsia in mice causes a reduced blood volume presumably secondary to loss of plasma through capillary walls made permeable by the rickettsial toxin.[13] One might expect that toxins of the Chlamydozoaceae will similarly increase the permeability of endothelial membranes. It is realized that changes in intraocular pressure associated with inflammations must also be considered as a factor in the formation of corneal edema. However, from experience with bacterial infections of rabbit eyes, we are not inclined to ascribe the severe degree of corneal edema seen in this experiment to inflammation.

SUMMARY

1. The virus of herpes simplex infected the following kinds of cells in the experimentally infected rabbit eye: all neural elements of the retina, fibroblasts, radial and circular muscle of the iris, muscle of ciliary body, pigmented and nonpigmented epithelium of the ciliary body, and macrophages. A single endothelial cell contained an inclusion.

The changes observable grossly in an infected eye were relatively inconspicuous in spite of the abundance of susceptible cells in the intraocular tissues.

Many of the highly susceptible retinal cells remained uninfected in the most severely infected eyes.

2. The virus of feline agranulocytosis infected the following kinds of cells

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in the experimentally infected cat's eye: fibroblasts, bipolar cells of visual retina, and pigmented epithelium of visual retina and ciliary body. This virus caused severe and often fatal systemic infection of intraocularly inoculated cats. However, the eyes on external examination in the living animal showed no macroscopic evidence of the intraocular infection that occurred.

3. Ornithosis virus injected in large dosage into the eyes of three rabbits caused a violent infection with conjunctival exudate, great thickening and opacity of the cornea and acute inflammation of the tissues near the filtration angle.

Inclusion bodies showed that macrophages, fibroblasts and a small number of endothelial cells of the inner surface of the cornea were infected. It was suggested that an important factor in the pathogenesis of the severe corneal edema was probably toxicity of virus released into the anterior chamber from infected cells, and consequent increased permeability of the endothelial membrane on the inner surface of the cornea.

THE SENSITIVITY OF *GAFFKYA TETRAGENA* STRAINS TO PENICILLIN AND STREPTOMYCIN

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Though *Gaffkya tetragena* is often regarded as a harmless saprophyte, it may on occasion produce serious infections which are generally characterized by septicemia, with localization producing arthritis and meningitis rather common.[1] Fulminating cases may terminate fatally as early as the 7th day,[2] while chronic cases may run a clinical course of as long as 7 months.[3] The mortality rate from 44 cases of septicemia was reported by Reimann[1] to be 50%. According to the same investigator, a total of more than 170 cases of infection with *G. tetragena* was reported in European literature prior to 1934. Cases in America, though rare, have been reported by Griewe and coworkers,[4] Steele,[5] Tobin,[6] Goetchius and Lawrence,[7] and Wynne.[8] We agree with Reimann[1] that such infections are probably more common than the literature indicates, since the organism is apt to be mistaken for a micrococcus.

To our knowledge no reports have appeared on the sensitivity of *G. tetragena* to streptomycin, and only one concerning its behavior toward penicillin. A strain isolated from a patient with septicemia was found by Goetchius and Lawrence[7] to require 5000 units of penicillin per ml for growth inhibition. Recently one of us (Wynne[8]) succeeded in isolating this organism from the blood stream in an undiagnosed febrile case of three months' standing which had been treated unsuccessfully with penicillin. Unfortunately the strain was lost before its in vitro resistance to penicillin was determined, but it failed to grow in the presence of 1 unit per ml of streptomycin. The present investigation represents a study of the sensitivity of 6 strains of *G. tetragena* to penicillin and streptomycin.

EXPERIMENTAL

Of the 6 *G. tetragena* strains employed, 2 (6007a and 6007b) were obtained from the American Type Culture Collection; 2 (951 and 7501) from the National Collection of Type Cultures, London; one (U. T.) from the Department of Bacteriology, University of Texas; and one (284) was isolated from a throat culture. Morphological and biochemical characteristics of all strains conformed satisfactorily to those in *Bergey's Manual*.[9]

The potassium salt of penicillin G obtained from Eli Lilly and Company, Indianapolis, was employed throughout these studies. Streptomycin used was obtained as the calcium chloride complex from Merck and Company, Rahway, New Jersey. Difco nutrient broth and nutrient agar, both at pH 6.8, were used to test penicillin sensitivity. For determining sensitivity to streptomycin, Difco nutrient broth at pH 7.9 or the special streptomycin assay agar of Loo and coworkers[10] was employed. The drugs were added

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aseptically after sterilization of the test mediums. Incubations were at 37 C.

Sensitivity to both antibiotics was determined by the well-known broth dilution and agar dilution plate methods. In the broth method the concentrations of penicillin or streptomycin employed were 0.0009, 0.0018, 0.0037, 0.0075, 0.015, 0.03, 0.06, 0.125, 0.25, 0.50, 1 and 10 units per ml in a final volume of 1.5 ml per tube. Since the *G. tetragena* strains grew at different rates, 72-hour broth cultures were diluted with several (usually 3 to 4) volumes of sterile water in such a way as to obtain approximately the same degree of turbidity in all strains. One drop per tube of these diluted suspensions was then employed as the inoculum. Incubation was from 24 to 48 hours. Typical results in table 1 indicate that all strains were more sensitive to streptomycin than to penicillin.

In the agar dilution plate method, the final concentrations of streptomycin employed were 0.1, 0.25, 0.5 and 1 unit per ml, while penicillin was used at levels of 0.01, 0.05, 0.1, 0.25, 0.5, 1 and 2 units per ml. Sectors of plates were massively streaked from undiluted 72-hour broth cultures, and the incubation period was 96 hours. In all cases resistance to both drugs was somewhat higher than in the broth method (table 1). Though 4 of the strains were slightly more sensitive to streptomycin than to penicillin, the differences in sensitivity to the two antibiotics were less marked by this method than by the broth method.

TABLE 1.—*Initial sensitivity of* G. tetragena *to streptomycin and penicillin by broth dilution and agar dilution plate methods.*

Strain	Inhibiting concentration of antibiotic, units/ml			
	Broth method		Agar plate method	
	Strepto-mycin	Penicillin	Strepto-mycin	Penicillin
6007-a	0.03	0.5	1.0	2.0
6007-b	0.03	0.5	1.0	1.0
U. T.	0.06	1.0	1.0	2.0
951	0.0075	0.03	0.5	1.0
7501	0.015	0.06	1.0	1.0
284	0.03	0.06	0.25	1.0

In an effort to develop streptomycin-resistant strains, 96-hour broth cultures were prepared from colonies appearing on the maximum concentrations of the antibiotic allowing growth. These cultures were then streaked to sectors of plates containing 0.1, 0.25, 0.5, 1, 2, 4, 8, 20, 50, 100, 200, 400, 800, and 1000 units per ml. Heavy suspensions were prepared in distilled water from colonies appearing after 3 to 4 days on the maximum tolerated concentration, and these were massively subcultured to a second series of plates. Results in table 2 show that although resistance was acquired at varying rates, all strains except 284 became resistant to 1000 units per ml in from 2 to 5 transfers in the presence of streptomycin. Our results are in substantial agreement with the numbers of transfers reported necessary to secure a similar tolerance of 12 strains of shigellae by Klein and Kimmelman.[11]

11. Klein, M. and Kimmelman, L. J. 1946, J. Bact. **52:** 471–479.

TABLE 2.—*Development of streptomycin resistance in* Gaffkya *strains (reported as maximum concentration allowing growth in units per ml).*

Strain	Number of transfer in presence of drug					
	Initial	1	2	3	4	5
6007-a	0.5	0.5	1.0	2.0	1000*	
6007-b	0.5	0.5	20	50	400	1000
U. T.	0.5	400	1000			
951	0.25	0.5	8.0	20	1000	
7501	0.5	1.0	8.0	20	1000	
284	0.1	0.25	4.0	4.0	4.0	4.0

* Highest concentration tested.

Repeated attempts to secure penicillin-resistant strains by a similar technique were uniformly unsuccessful. In this connection it is of interest to note that several investigators have reported failures to observe penicillin resistance in *Neisseria gonorrhea*, even in vivo.[12] Quite recently 6 strains of *Actinomyces bovis* have been reported by Boand and Novak[13] to develop little or no resistance to penicillin in 32 transfers, while all showed at least a 250-fold increase in resistance to streptomycin in 10 transfers.

To test the possibility of cross resistance, two strains tolerating 1000 units per ml of streptomycin were massively streaked to plates containing graded amounts of penciillin. However, in both cases there was an actual slight decrease in penicillin resistance compared to that of the parent strains.

DISCUSSION

The somewhat greater initial sensitivity of strains of *G. tetragena* to penicillin and streptomycin obtained with the broth dilution method compared to that observed with the agar dilution plate method may have been due to the larger inoculums used in the latter procedure. Presumably these larger inoculums may have contained cells of greater resistance (see Klein and Kimmelman[11] for discussion of this point).

Although all strains tested exhibited a greater initial sensitivity toward streptomycin than to penicillin, the failure to observe development of in vitro penicillin resistance might indicate this antibiotic to be the drug of choice in the treatment of *G. tetragena* infections. If streptomycin is used, an adequate initial dosage would seem of possibly paramount importance.

SUMMARY

All of 6 strains of *Gaffkya tetragena* were inhibited by 1 unit per ml or less of penicillin in broth, and by 0.06 unit per ml or less of streptomycin. An agar dilution plate method showed somewhat higher resistance, with inhibitory concentrations ranging up to 2 units per ml for penicillin and 1 unit per ml for streptomycin.

Five of the 6 strains became resistant to 1000 units per ml of streptomycin within 5 transfers on agar. Attempts to demonstrate resistance to penicillin were unsuccessful.

12. Willcox, R. R. 1949, Bull. Hyg. **24:** 99–111.
13. Boand, A. and Novak, M. 1949, J. Bact. **57:** 501–508.

THE COMPLEMENT FIXATION INHIBITION TEST AND ITS APPLICATION TO THE DIAGNOSIS OF ORNITHOSIS IN CHICKENS AND IN DUCKS

I. PRINCIPLES AND TECHNIQUE OF THE TEST

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For the last 15 years, laboratory diagnosis of psittacosis and similar diseases has been sucessfully aided by the complement fixation test. For clinical use it is a practical device because among other reasons, isolation of the virus is often difficult and expensive. It has gained in importance in the last few years when diagnosis has provided a direction for effective therapy. In epidemiological and experimental work the test has effectively detected antibodies in the serum of infected psittacine birds (Meyer and Eddie, 1939; Meyer et al, 1939), pigeons (Meyer, 1941; Meyer et al, 1942; Eddie and Francis, 1942), seashore birds (Pollard, 1947), guinea pigs (Bedson, 1932; Meyer and Eddie, 1939), goats (Meyer and Eddie, 1939), monkeys, rabbits and man. Since psittacine birds were considered to be the reservoir of the virus most threatening to human beings, the test has served an important function.

Recently, epidemiologic studies have produced evidence that the ornithosis virus is also harbored by chickens and ducks and may be transmitted by these birds to man. The serologic reaction of these fowl to the complement fixation test has only very seldom been positive in the presence of infection, and then only in titers of 1:2 to 1:16 (Eddie, 1945–46). Studies reported in two of this series of three papers were instigated to find a means of revealing ornithotic infection in these common barnyard fowl.

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PRELIMINARY EXPERIMENTS

One can propose at least four reasons for the negative reaction to the complement fixation test with certain immune serum: (1) The antibody might be unable to fix guinea pig complement (the one commonly used) but readily fix complements of other animal species. (2) The immune serum might have nonspecific hemolytic properties. (3) The complement might be destroyed by some influence of the immune serum. (4) The antibody might have a structure which does not allow fixation of any complement by the antigen-antibody complex. The first experiments were carried out to determine which of the four possible reasons provided a valid explanation of the lack of complement fixation by chicken immune serum.

The method employed for the complement-fixation test was that described by Meyer and Eddie (1939), but instead of mouse spleen, the yolk sac cocto antigen (6BC strain) was used (Meyer and Eddie, 1948).

1. Different complements.—According to Noguchi and Bronfenbrenner (1911), a chicken amboceptor directed against human red cells allowed hemolysis only if chicken or hog complement was used. Since an amboceptor is a component of immune serum and therefore comparable to the ornithosis chicken immune serums used for these experiments, the findings of Noguchi and Bronfenbrenner made it seem possible that chicken or hog complements would prove more successful than guinea pig complement. Both of these and several others (human, rabbit, cattle and pigeon serum) had been used by Eddie and fixation had not occurred. The chicken complement had to be used undiluted due to its low titer; the hog complement could be diluted 1:10. Diluted cotton rat (*Sigmodon hispidus*) complement (1:20 to 1:30) was tried with the same negative results.

2. *Nonspecific hemolysis by chicken serums.*—

Low dilutions (1:2 to 1:8) of the serums were made in the attempt to determine whether nonspecific hemolytic reaction is characteristic of chicken serum. Chicken immune serum, complement and unsensitized sheep red cells were used. Only very few inactivated chicken serums effected lysis, and none fixed complement in the presence of antigen. Therefore, it cannot be assumed that chicken hemolysins directed against sheep cells brought about the complete hemolysis in all the tubes.

3. Destruction of complement.—Inactivated (30 minutes at 56 C) normal and immune serums were incubated at 37 C together with complement, which was afterward used in a complement fixation test. The complement did not manifest abnormal deterioration except the normal titer decrease due to incubation at 37 C.

It seemed obvious after the preceding observations that some property of chicken antibody or some property of the immune aggregate resulting from the rapid union of the antibody and the ornithosis (psittacosis) antigen did not allow fixation of the complement.

A perusal of the literature discloses that horse serum possesses fixation-inhibiting properties. Calmette and Massel (1910) observed inhibition of complement fixation of immune horse serum by that of a calf vaccinated against *Mycobacterium tuberculosis*. Zinsser and Parker (1923) found that fixation could not be obtained with combinations of pneumococcus capsular polysaccharide and specific immune horse serum, although complement fixation did occur when the same substances were combined with specific immune rabbit serum. In 1935, Pittman and Goodner reported similar results for the specific polysaccharide and specific immune horse serums, although complement fixation did occur when the same substances were combined with specific immune rabbit serums. In 1935, Pittman and Goodner reported similar results for the specific polysaccharide of *Haemophilus influenzae*. A critical survey of the general mechanism of the complement fixation reaction with special reference to horse serum led Goodner and Horsfall (1936) to the recognition that complement is not fixed by immune aggregates resulting from the interaction of pneumococcus and type-specific immune horse serum. They furthermore confirmed the significant observation made by Zinsser and Parker that immune horse serum would completely block the fixation normally obtained with immune rabbit serum. This inhibition phenomenon is type specific. The horse serum per se is not inhibiting, but the phenomenon depends on the order in which the two antibodies react with the antigen.

On the basis of our preliminary work and the observations made by the workers cited, it seemed obvious that certain immune serum aggregates fail to fix complement in the presence of suitable antigens and, in addition, inhibit complement fixation by antiserum of other animal species. It appeared not unlikely that the serum of ornithosis-infected chickens might be of this type, and in the subsequently reported work, the correctness of the assumption was confirmed. The inhibition phenomenon thus provided the basis for the diagnostic test which we had hoped to find when these studies were undertaken early in 1947.

Rice (1947, 1948a and b) and Hilleman and Helmold (1949) also reported application of this variant from the usual complement fixation reaction with *Salmonella pullorum*, pneumococcus and viruses of the psittacosis group.

COMPLEMENT FIXATION INHIBITION TEST

Complement fixation test.—A modification of the complement fixation test used by Meyer and Eddie (1939 and 1948) was applied.

Antigen.—The yolk sac cocto antigen (6BC strain) was used exclusively. It was titrated with a complement-fixing antipsittacosis pigeon serum of high titer. In the subsequent test proper, 1 unit of antigen was used. This unit was determined for each test by the standard titration technique with an antipsittacosis pigeon serum of high complement-fixing titer. One unit is the highest dilution of antigen giving complete inhibition of hemolysis.

Sensitized sheep red cells.—These were kept in modified Alsever's solution (Bukantz, Rein and Kent, 1946) until a further modification was adopted which yielded less coagulation and retarded hemolysis of the stored sheep blood:

Glucose	2.05 g
Sodium citrate	1.2 g
Sodium chloride	0.42 g
Water enough to make 100 ml	
Adjust to pH 6.1 with citric acid	

The cells were washed 4 times with the buffer, using 5 or 6 times the volume of cells. Subsequently, a 2% red cell sus-

pension was made up which contained 2 units of rabbit hemolysin.

Buffer.—In previous experiments, phosphate-buffered saline (pH 7.4) was used. Later a veronal buffer (Mayer et al, 1946) came to be preferred because it yielded higher titers of amboceptor and complement:

Sodium chloride	85.00 g
5,5-diethyl barbituric acid (veronal)	5.75 g
Sodium 5,5-diethyl barbiturate	3.75 g
$MgSo_2 \cdot 7H_2O$	2.028 g
$CaCl_2 \cdot 2H_2O$	39.2 mg

Distilled water up to 2000 ml

For use: 1 part diluted with 4 parts distilled water

Complement.—This consisted of at least 10 pooled male guinea pig serums and was stored in the frozen state. The titration was carried out as designated in table 1. A parallel titration without

TABLE 1.—*Complement titration with antigen.*

Tube no.	Complement (1:30)	Diluted antigen (1 unit)	Buffer
1	0.08	0.25 ml	0.67
2	0.10	0.25 ml	0.65
3	0.12	0.25 ml	0.63
4	0.14	0.25 ml	0.61
5	0.16	0.25 ml	0.59
6	0.18	0.25 ml	0.57
7	0.20	0.25 ml	0.55

antigen was also run at the same time. After 2 hours at 37 C, sheep red cells (0.5 ml) previously sensitized for 10 minutes at 37 C with 2 units of rabbit hemolysin were added, and the mixture was incubated for 1 hour at 37 C. Two exact units of complement were used in the test.

Tables 2 and 3 give examples of the technique used in the complement fixation inhibition test.

Complement-fixing pigeon immune serum.—Serum was obtained either from birds of lofts known to harbor the virus or from pigeons immunized with various strains (1 to 3 intramuscular injections of 0.5 to 1.0 ml of a 20% yolk sac culture). Only serum with titers of 1:32 or higher were used, and which in the high dilutions gave no fixation in presence of normal yolk sac antigen.

TABLE 2.—*Selection of pigeon serum. Titration to determine the highest dilution giving complete fixation with the smallest amount of antigen.*

0.25 ml diluted pigeon serum	Antigen dilution (0.25 ml of each) 1:32 (4 units)	1:64 (2 units)	1:128 (1 unit)	Add 2 exact units of complement. Incubate 2 hours in 37 C water bath, add sensitized cells and incubate in 37 C water bath for 1 hour.
1:64	4+	4+	2+	
1:96	4+	4+	2+	
1:128	3+	4+*	2+	
1:192	1+	2+	±	

* The pigeon serum dilution to be used in the test is 0.25 ml of that dilution which gives a 4+ reaction with the smallest amount of antigen. Example: antigen 1:64 and pigeon serum 1:128. Therefore, pigeon serum in a dilution of 1:128 is used in the test.

Chicken serum.—Experimentally, the immune serum was produced by injecting 1 to 3 cc of yolk sac cultures (20% suspensions) intramuscularly or intraperitoneally into adult roosters. One to 10 or more injections were made. Blood was withdrawn and allowed to clot overnight at room temperature, and the serum was subsequently kept at 5 C.

Inactivation of serum.—All serums were inactivated at 60 C for 30 minutes; this proved more successful in overcoming the sometimes marked anticomplementary action of chicken serum than incubation at 56 C. To minimize hazy precipitations of serum protein, serum was diluted 1:4 (pigeon) or 1:2 (chicken) previous to inactivation. Rarely, heavy protein precipitates occurred if inactivation took place at 60 C, and they were observed only if the serum was contaminated. Tests with such serum were not considered conclusive and were repeated with serum incubated at 56 C.

Titration of pigeon serum.—After the complement titration, a high-titered, complement-fixing pigeon serum was titrated against different amounts of antigen (approximately 4, 2, 1 and ½ units). The purpose of this titration is the selection of the serum dilution that will provide the highest sensitivity of the subsequent complement fixation in-

hibition test, that is, the highest dilution that gives a 4+ reaction (1 serum unit).

The procedure is: (1) Make a serum dilution—1:16, 1:24, 1:32, 1:48, 1:64, 1:96, 1:128 and so forth. (2) To 0.25 ml of each serum dilution, add 0.5 ml of complement (dilution containing 2 exact units) and 0.25 ml of antigen. (3) Incubate the mixture at 37 C for 2 hours. (4) Add 0.5 ml of sensitized sheep red cells. (5) Incubate at 37 C for 1 hour. (6) Select the highest serum dilution which gives a 4+ reaction for use in the complement fixation inhibition test.

Procedure for the complement fixation inhibition test.—(1) Make twofold dilutions of the serum to be tested, starting with 1:2. (2) Mix 0.25 ml each of diluted serum, antigen (1 unit), complement (2 exact units) and pigeon serum (1 unit). (3) Incubate the mixture at 37 C for 2 hours. (4) Add 0.5 ml of sensitized sheep red cells. (5) Incubate the mixture at 37 C for 1 hour.

The degree of complement fixation is indicated in the usual way: 4+, complete fixation; 3+, partial fixation (90% or more); 2+, partial fixation (50% to 90%); 1+, partial fixation (10% to 50%); ±, little fixation (5% or less). However, the interpretation of the test is directly opposite. In the complement fixation test, complete fixation (no hemolysis) indicates a positive result. In the complement fixation inhibition test, complete hemolysis (no fixation) indicates a positive result. The inhibition titer of the chicken serum is considered positive in the highest dilution in which fixation of complement is

TABLE 3.—*Setting up the test.*

Tube no.	0.25 ml of serum to be tested in dilution of	Pigeon serum diluted as previously titered (1:128)	Antigen, 1 unit	Complement, 2 exact units	Buffer	Sensitized cells—2 units hemolysin in 2% sheep cells	Readings	Readings
		ml	ml	ml		ml		
1	1:2	0.25	0.25	0.25	Shake well. Preliminary incubation 2 hr. 37 C water bath or 9 hr. 6–8 C followed by 10 min. 37 C	0.5	Shake well. Secondary incubation 37 C water bath 1 hr.	−
2	1:4	0.25	0.25	0.25		0.5		−
3	1:8*	0.25	0.25	0.25		0.5		±
4	1:16	0.25	0.25	0.25		0.5		2+
5	1:32	0.25	0.25	0.25		0.5		4+
				Complement fixation controls				
6	1:2	None	0.25	0.25	0.25 Shake well. Preliminary incubation 2 hr. 37 C water bath or 9 hr. 6–8 C.	0.5	Shake well. Secondary incubation 37 C water bath 1 hr.	−
7	1:4	None	0.25	0.25	0.25	0.5		−
8	1:8	None	0.25	0.25	0.25	0.5		−
				Serum controls				
9	1:2	None	None	0.25	0.5	0.5	Shake well. Secondary incubation 37 C water bath 1 hr.	−
10	1:4	None	None	0.25	0.5	0.5		−
11	1:8	None	None	0.25	0.5	0.5		−
				Lysin controls			(2% cells only)	
12	1:2	None	None	0.25	1.0	None	0.25	4+
13	1:4	None	None	0.25	1.0	None	0.25	4+
14	1:8	None	None	0.25	1.0	None	0.5	4+
				Pigeon serum controls				
15	None	0.25 of 1:128	0.25 of 1:64	0.25	0.25	0.5		4+
16	None	0.25 of 1:192	0.25 of 1:64	0.25	0.25	0.5		2+
17	None	0.25 of 1:256	0.25 of 1:64	0.25	0.25	0.5		±
18	None	0.25 of 1:128	0.25 of 1:128	0.25	0.25	0.5		2+
19	None	0.25 of 1:192	0.25 of 1:128	0.25	0.25	0.5		±
20	None	0.25 of 1:256	0.25 of 1:128	0.25	0.25	0.5		−

* This titer is the highest dilution which still gives complete or nearly complete inhibition.

completely or almost completely inhibited (complete or almost complete hemolysis).

Controls.—In addition to the complement fixation inhibition test proper, four control tests are made to check the accuracy of the test:

1. The standard complement fixation test of the chicken serum is run to find if, by chance, it will fix complement. In the course of these experiments such serum was not found among hundreds of samples. Components: chicken serum in low, twofold dilutions, complement, 1 unit of antigen and sensitized cells.

2. A second titration of the pigeon serum is made, using standard complement-fixing technique "pigeon serum control." The results must be identical with those of the preliminary titration. Components: pigeon serum, complement, antigen and sensitized cells.

3. The chicken serum is tested for hemolysins against sheep cells "hemolytic control." If they are present, hemolysis in the test proper does not indicate inhibition of fixation. Components: chicken serum in low, twofold dilutions, complement and unsensitized sheep cells.

4. The chicken serum is tested also for anticomplementary properties. Components: chicken serum in low, twofold dilutions, complement and sensitized cells.

An example of the complement fixation inhibition test is given in table 3.

Influence of quantity of antigen and pigeon antibody.—An inhibiting duck serum was titrated by the complement fixation inhibition method using different dilutions of antigen and pigeon serum. The result of the test is recorded in table 4. This shows the very considerable decrease of inhibitory titers if an excess of either antigen or pigeon serum

TABLE 4.—*Influence of quantity of antigen and pigeon antibody upon the inhibitive titer of a duck serum.*

Pigeon serum dilutions	Duck serum dilutions						Antigen dilutions
	1:8	1:16	1:32	1:64	1:128	1:256	
1:16	4+	4+	4+	4+	4+	4+	1:16
	4+	4+	4+	4+	4+	4+	1:32
	4+	4+	4+	4+	4+	4+	1:64
	4+	4+	4+	4+	4+	4+	1:128
	3+	3+	4+	4+	4+	4+	1:256
1:32	4+	4+	4+	4+	4+	4+	1:16
	4+	4+	4+	4+	4+	4+	1:32
	4+	4+	4+	4+	4+	4+	1:64
	3+	4+	4+	4+	4+	4+	1:128
	3+	3+	4+	4+	4+	4+	1:256
1:64	3+	4+	4+	4+	4+	4+	1:16
	3+	4+	4+	4+	4+	4+	1:32
	3+	3+	4+	4+	4+	4+	1:64
	2+	3+	4+	4+	4+	4+	1:128
	1+	2+	3+	3+	4+	4+	1:256
1:128	±	3+	3+	3+	4+	4+	1:16
	–	2+	4+	4+	4+	4+	1:32
	–	1+	3+	4+	4+	4+	1:64
	–	±	3+	4+	4+	4+	1:128
	–	–	±	3+	3+	4+	1:256
1:256	–	–	1+	3+	3+	3+	1:64
	–	–	–	2+	3+	3+	1:128
	–	–	–	±	3+	3+	1:256

Pigeon serum dilutions							Antigen dilutions
1:16	1:32	1:64	1:128	1:256	1:512	
4+	4+	4+	3+	–	–	1:16
4+	4+	4+	4+	±	–	1:32
4+	4+	4+	4+	3+	–	1:64
4+	4+	4+	4+	3+	±	1:128
4+	4+	4+	4+	3+	1+	1:256

is used. The highest titer is obtained in a system that contains not more than 1 unit of both antigen and pigeon immune serum (antigen, 1:256, pigeon serum, 1:256 in the recorded test). The highest sensitivity of the test is achieved if in the fixation control only one tube shows 4+ fixation, that which contains

components.—Two methods of incubation were used in a test with identical reagents. Several serum specimens, obtained from a duck farm where birds had been proven to carry the virus, were diluted into two series of tubes. One set was tested by the method described under Procedure. To the other set, only

TABLE 5.—*Influence of incubation of serum with antigen.*

Duck serum	Method of incubation	Serum dilutions					Virus isolated
		1:4	1:8	1:16	1:32	1:64	
100 MG	*Water bath	2+	2+	2+	3+	4+	+
	†Ice box	1+	1+	2+	3+	4+	
103	Water bath	4+	4+	4+	4+	4+	0
	Ice box	4+	4+	4+	4+	4+	
W24	Water bath	–	1+	2+	3+	4+	+
	Ice box	–	±	1+	3+	3+	
W25	Water bath	3+	3+	3+	4+	4+	+
	Ice box	3+	3+	3+	3+	4+	
49W	Water bath	2+	3+	3+	4+	4+	S‡
	Ice box	3+	3+	3+	3+	3+	
W18	Water bath	1+	2+	3+	4+	4+	+
	Ice box	1+	2+	3+	3+	4+	
103 MC	Water bath	4+	4+	4+	4+	4+	0
	Ice box	4+	4+	4+	4+	4+	
W30	Water bath	–	–	1+	3+	3+	+
	Ice box	–	–	±	1+	3+	
2W	Water bath	4+	4+	4+	4+	4+	0
	Ice box	3+	4+	4+	4+	4+	
46W	Water bath	3+	3+	3+	4+	4+	0
	Ice box	2+	2+	3+	3+	4+	

* Duck serum, antigen, complement and pigeon serum added in order given, incubate 37 C water bath for two hours, add sensitized cells, additional one hour water bath.
† Duck serum and antigen incubate at 5–7 C ice box over night. Preliminary 37 C water bath for 30 minutes, add complement and pigeon serum, water bath 2 hours, add sensitized cells, water bath one hour.
‡ S = suspected. Animal passage was not possible.

the antigen and pigeon serum dilutions of the test proper. If more than 1 unit of either reagent has been used, the titers of the fowl serum are lowered or eventually the serum reacts in a manner indicative of a negative result. If less than 1 unit has been used, the titer of the fowl serum is generally a little raised, but the reading has to be made with care, considering that the fixation control does not show complete (4+) fixation. The degree of hemolysis as observed in the fixation control must not be misinterpreted in the test proper as partial inhibition by the fowl serum.

Influence of incubation of fowl serum and antigen prior to addition of the other the antigen was added, and the mixture was incubated overnight at 5 C, and for 30 minutes at 37 C on the second day. Thereafter complement and pigeon serum were added. The pigeon-serum titration was carried out on the second day with antigen which had been incubated overnight and at 37 C, like the tubes of the test proper. The result of the test with 10 samples is recorded in table 5. This shows that preliminary incubation of fowl serum together with antigen results only in a slight rise of inhibition titers. Serums completely negative by the "standard" method (7, 9, 10) remain negative if tested by the incubation method. Therefore, it does not seem

necessary to give preference to the latter; there is no great danger of missing positive results if the standard method is employed.

Pigeon serum is not completely satisfactory because only a limited amount can be obtained (rarely more than 9 or 10 ml) by bleeding the bird out from the heart. Moreover, many birds do not respond to immunization with complement-fixing antibodies, probably because they have had previous contact with the virus and the inhibitory property has developed (Karrer et al, 1950). For these reasons guinea pig immune serum was tried in earlier experiments but was not found able to replace pigeon serum. With the serum of most guinea pigs inhibition was not observed, although the chicken serum used in these experiments inhibited the complement fixation if pigeon serum was used instead of guinea pig serum. In many instances, guinea pig serum could be inhibited only by certain chicken immune serum samples but not by others. In every instance the chicken serum has shown inhibitory activity if a pigeon serum was used. In only one instance did a guinea pig serum react in a way comparable with pigeon serum; this serum was obtained from an animal immunized with the Petaluma duck strain of the virus. Table 6 shows the results obtained with guinea pig serum in comparison with pigeon serum.

It is obvious that the complement-fixing power of guinea pig serum usually is not as easily inhibited as that of pigeon serum. Inhibition might have been greater if the chicken serum had been used in low dilutions, but the uniformly high inhibition titers obtained in the presence of pigeon serum are paralleled in only one instance (Petaluma strain). Serum of guinea pig 130 was tested twice, one giving 4+ fixation, the other 2+ in a dilution of 1:256. Whereas in the first series, the chicken serum did not show inhibition, the second series did (fixation control, 2+), which confirms the results summarized in table 4. This also gives an indication that the 50% hemolysis method as employed by Rice will be of higher sensitivity in the complement fixation inhi-

TABLE 6.—*Comparison of inhibition reaction with guinea pig and pigeon serum.*

Guinea pig	Guinea pigs infected with	Chicken immune serum									Fixation controls
		1 (Mulay)			2 (SF)			3 (Francis)			
		Dilution									
		1:64	1:128	1:256	1:32	1:64	1:128	1:8	1:16	1:32	
6	Louisiana	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
112	Louisiana	4+	4+	4+	4+	4+	4+		4+	4+	4+
130	Detroit	4+	4+	4+	4+	4+	4+		4+	4+	4+
130	Detroit	±	1+	2+				−	−	−	2+
149	Francis	4+	4+	4+	4+	4+	4+		4+	4+	4+
102	Endo	2+	3+	3+				4+	4+	4+	4+
35	Louisiana	4+	4+	4+				−	±	3+	4+
42	Louisiana	4+	4+	4+				−	−	2+	4+
37	Bar Harbor	−	±	3+	−	3+	4+		4+	4+	4+
151	Louisiana	3+	3+	4+	3+	4+	4+		4+	4+	4+
65	Stockholm	3+	3+	4+				3+	4+	4+	4+
53	Louisiana	3+	3+	3+	3+	3+	3+		3+	3+	3+
100	Endo	3+	3+	3+				2+	3+	3+	4+
	Petaluma	−	−	1+	−	±	3+	1+	3+		4+
Pigeon 16545	Pigeons (all naturally infected)	1+	1+	1+	−	−	−				3+
NY2		−	±	1+	−	1+					3+
304		−	−	1+	−	±	1+				3+
302		1+			−						4+
291 & 299		−			−						4+
NY1		−	−	−	−	−					2+

bition test than the 100% method used in these studies.

Serum from several ornithosis-infected patients has been used as the serum. In most instances, striking titer differences were not observed, contrary to the guinea pig serum (table 7).

Table 7 shows both rather uniform

TABLE 7.—*Inhibition reaction of chicken serum with pigeon serum.*

Chicken Infecting strain	*Pigeon serum	Chicken serum dilutions and titers							
		1:4	1:8	1:16	1:32	1:64	1:128	1:256	Controls
				Similar inhibition					
Chick B	1	−	−	−	−	±	+		3+
	2	±	−	−	−	1+	2+		
	3	−	−	−	−	±	1+		
	4	±	±	±	±	±	±		
Petaluma duck	1	−	−	−	1+	3+	3+		3+
	2	−	−	±	1+	2+	3+		
	3	−	−	−	±	1+	2+		
	4	−	−	±	1+	2+	2+		
Seattle parakeet	1	−	−	±	2+	3+	3+		3+
	2	−	−	1+	1+	3+	3+		
	3	−	−	−	±	1+	2+		
	4	−	−	±	1+	2+	3+		
Bar Harbor mouse	1	±	±	1+	2+	3+	3+		3+
	2	±	±	1+	2+	3+	3+		
	3	±	±	1+	3+	2+	3+		
	4	±	1+	1+	2+	2+	3+		
Lympho-granuloma venereum	1	−	−	1+	2+	3+	3+		3+
	2	−	−	1+	1+	2+	3+		
	3	−	−	1+	2+	3+	3+		
	4	−	±	2+	2+	3+	3+		
Louistana human	1	−	−	−	1+	2+	3+		3+
	2	−	−	±	1+	2+	2+		
	3	−	−	−	±	2+	2+		
	4	−	−	±	1+	2+	3+		
				Slight difference of inhibition					
Francis mouse	5			−	−	1+	3+		4+
	6			±	2+	3+	3+		
	7			±	1+	2+	3+		
	8			±	1+	2+	3+		
Mulay parakeet	5					−	1+	2+	4+
	6					±	2+	3+	
	7					−	−	−	
	8					−	−	−	
Duck	5				−	−	1+	3+	4+
	6				−	1+	3+	3+	
	7				−	−	−	1+	
	8				−	−	−	±	

* 1–2 naturally infected pigeons; 3–4 pigeon serum infected with Petaluma duck strain; 5–8 naturally infected.

fixing serum, but on a very limited scale. Occasionally, inhibition could be observed. Since this type of human serum is difficult to obtain in sufficient amounts, it is not considered to be of great value for the test. It did not give uniform results in the few instances in which it was used.

Different pigeon serums gave slightly different inhibition titers if employed in parallel tests with the same chicken inhibition titers obtained with serum from 4 different pigeons and some considerable titer differences. Thus, serum from pigeons 5 and 6 give, on the whole, higher inhibition titers than those from pigeons 1 and 2. Nevertheless, the differences are smaller by far than those observed with guinea pig serum. Great care was taken to use 1 unit of pigeon serum in all these titrations in order to make the test equally sensitive for all

pigeon serums, because the amount of pigeon serum also influences the inhibition titers (table 4).

With two other pigeon serums, the results were less uniform (table 8). Pigeon serum N. Y. 2 yields definite inhibition titers in 3 cases in which serum 16545 titers were negative for the same duck serums (ducks 4, 10 and 11). With the other serums, the inhibition titers were higher in the presence of pigeon serum N. Y. 2.

in epidemiological surveys of fowl populations, it is of little importance if a few faintly positive results are missed because the test is not highly sensitive.

We have used the term "complement fixation inhibition test," rather than "indirect complement fixation test" (the name proposed by Rice), since the former term describes more accurately the test and also forms a parallel to other terms like "hemagglutination inhibition test." The fixation of complement in

TABLE 8.—*Inhibition reaction of duck serum with pigeon serum.*

Duck serum	Pigeon serum (birds naturally infected)									
	*NV 2					†HC 16545				
	Serum titers									
	1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16	1:32	1:64
46W	2+	3+	3+	3+	3+	3+	3+	3+	4+	4+
W25	±	−	±	2+	3+	3+	3+	3+	4+	4+
W30	1+	−	−	1+	3+	−	−	1+	3+	3+
49W	−	−	1+	3+	4+	2+	3+	3+	4+	4+
2W	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+
W18	±	1+	2+	4+	4+	1+	2+	3+	4+	4+
103MC	2+	3+	3+	4+	4+	4+	4+	4+	4+	4+
96MC	−	−	±	2+	3+	3+	4+	4+	4+	4+

Fixation control: 4+.
* NV 2—pigeon from New York.
† HC 16545—pigeon from California.

DISCUSSION

This first paper has been limited to a consideration of the different factors involved in the complement fixation inhibition test. Experiments which prove the specificity of the test will be reported in two other papers (Karrer et al, 1950; Karrer and Hammon). Since the main purpose of these studies was a practical one, namely, to develop a suitable in vitro test of avian immune serum, less attention has been paid to the technique itself. The presented "standard" technique (without preliminary incubation of antigen and fowl serum) does not give high inhibition titers and is very crude compared with the 50% method employed by Rice. But it represents an easy method, little more elaborate than the straight complement fixation test. Moreover, a more accurate technique is not necessary for practical use, since, this test is indeed not the main phenomenon; it serves only to demonstrate another factor—a poorly understood property of the specific antibody in certain avian serums. There is no great danger of confusion with Caulfield's inhibitive test, as Rice (1948b) feared, if one knows about the principles of both methods.

In accordance with Rice's (1948a) more elaborate investigations, it was found that the inhibitive titers depend largely on the amounts of fixing serum and of antigen employed. The smaller degree of sensitivity of the complement fixation inhibition test (100% method) compared with the inhibition test as developed by Rice (50% method) is expressed by these amounts of fixing serum and antigen. Thus, Rice (1948b) could recommend the use of 10 to 15 fixing units of antigen and 6 to 8 fixing units of serum and still get satisfactory

inhibition of complement fixation by fowl immune serums, whereas in these studies 1 unit had to be chosen for each reagent in order to get reasonable inhibition results. The danger of obtaining false positive results in this sensitive system with fowl serums of hemolytic properties (Rice, 1948b) is not considerable, if "hemolytic controls" (fowl serum, complement and unsensitized cells) are made.

Preliminary incubation of antigen and fowl serum did not increase the titers very considerably, even if extended overnight in the refrigerator. This does not exclude the value of the "incubation method," which was later found very satisfactory for the encephalitis system (Karrer and Hammon).

Whereas Rice (1947) used serum of rabbits, pigeons or guinea pigs as fixing serums, only pigeon serum was found universally satisfactory in the reported experiments. This discrepancy is perhaps caused by the difference in sensitivity of the 50% hemolysis method and the method presented in this paper. Serum of rabbits was not tried because these animals do not respond with high fixation titers to immunization with psittacosis-like viruses. On the basis of observations reported later (Karrer et al, 1950) regarding the inhibitive power of certain pigeon serum, it can be assumed, theoretically, that pigeons tend to develop antibodies which fix complement only loosely, and sometimes not at all. Their postulated loose fixing power is, therefore, inhibited more easily than that of guinea pig serum. The Petaluma duck strain is perhaps apt to produce immune reactions in guinea pig serum which allows inhibition similar to that of pigeon serum, because the guinea pig serum most suitable for the test originated from a guinea pig immunized against this strain (table 6) and because in numerous other tests the serum from this guinea pig was comparable with the easily inhibited pigeon serums.

Although most pigeon serums gave rather uniform titers for the same fowl serum, it is by no means certain that all serums are equally suitable for the inhibition test. The relationship of the infecting virus strain and the strain used to prepare the antigen may be important. In this study, the same strain was always used in the antigen. Strain-specific psittacosis antigens are not available. In later studies with the encephalitis group, the test proved to be rather strain specific (Karrer and Hammon). The titer discrepancies recorded in table 8 can perhaps be attributed to differences between the infecting and the antigen strains. Table 8 shows that three duck serums inhibit complement fixation in the presence of one pigeon serum, but not in the presence of another. Duck serum and pigeon serum N. Y. 2 were obtained from birds in New York State; pigeon 16545 serum was obtained from a bird in Hayward, California. Supposedly, the strain infecting the New York birds might differ considerably from the Hayward pigeon strain. This might explain the differences in the inhibition titers. Experimental proof of this hypothesis has not been established.

SUMMARY

A method (complement fixation inhibition test) is described which can be applied to the serologic diagnosis of ornithosis in domestic fowl. Serum of chickens immunized against different strains of psittacosis-like viruses inhibits the complement fixation which occurs in the presence of antigen and pigeon immune serum.

The importance of quantity of reagent (antigen and pigeon serum), incubation, and origin of the complement-fixing serum are discussed.

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THE COMPLEMENT FIXATION INHIBITION TEST AND ITS APPLICATION TO THE DIAGNOSIS OF ORNITHOSIS IN CHICKENS AND IN DUCKS.

II. CONFIRMATION OF THE SPECIFICITY AND EPIDEMIOLOGICAL APPLICATION OF THE TEST

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In the first paper of this series (Karrer et al, 1950) a method of detecting antibodies in the serum of ornithosis-infected chickens was described. The studies reported therein were continued and experiments were conducted to determine the specificity of this complement fixation inhibition test. Later, it was applied to an epidemiologic study.

The technique of this new test was fully described, but its specificity was not discussed. Ideally, a good serologic test should be specific for one certain disease or a related group of diseases and should be simple in technique and of high sensitivity. The last-mentioned two requirements are often difficult to meet in a single test. Thus, the 50% method of the complement fixation inhibition test (developed by Wadsworth and others, 1938) is more sensitive, but it also is more elaborate than the standard technique. The 100% method (Karrer et al, 1950) was preferred for this series of studies because one objective was its application in epidemiological surveys of fowl populations where high sensitivity is less important than simplicity, especially since it does not matter if, in the course of such studies, a few faintly positive results are missed because the test is less sensitive.

The problem of proving the specificity of the test was approached from two sides. First, in the laboratory, strains of psittacosis-like viruses were used to infect chickens, and subsequently the new method was used to test their serum. Secondly, field specimens from domestic fowl were tested by the same technique, and virus isolations attempted from seropositive birds. The latter was not expected to be conclusive because psittacosis virus isolations from fowl are known to be very difficult. The examination of field specimens was little apt, therefore, to prove the specificity of the test, but it was hoped that it would give an instructive picture of the probable infection rate among the tested small fowl populations (as measured by positive serologic reactions) once the value of the test had been proved. These results with field specimens are reported since we finally considered these experiments to be applications of the new test, rather than to be proof of its specificity.

Other matters of concern were the interval after infection before results become positive and the behavior of pigeon and human antiserum. Although the latter two have long been known to contain complement-fixing antibodies, they were now tested on a limited scale for inhibiting properties as well. These last experiments brought forth some altogether new findings which allowed conclusions regarding the nature of the inhibiting substance and its relation with the complement-fixing antibody.

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1. Experimental study of the specificity of the test

This experiment was originally planned in order to observe the influence of a chicken strain of *Miyagawanella psittaci* on health and growth of infected baby chicks. At the same time it helped to confirm the specificity of the complement fixation inhibition test.

The strain used to infect the chicks had been isolated from an approximately two-year-old hen obtained in 1947 from a ranch in Petaluma, California. Serum from this bird had inhibited fixation in dilutions of 1:2++++ and 1:4+. One year later the bird was bled out and the serum still inhibited complement fixation in the lowest dilution of 1:2++. The injection of organs and cloaca into mice resulted in the isolation of a psittacosis-like virus. Yolk sac cultures were highly virulent for white mice infected intranasally ($LD_{50} = <10^{-9}$), as well as intraperitoneally ($LD_{50} = 10^{-7.5}$) or intracerebrally.

The 79 white Leghorn baby male chicks used in the experiment were obtained from a hatchery at the age of two days. The only means of determining whether infection was present was the complement fixation inhibition test, although its diagnostic value at that time was unknown. Serologic reactions of only 4 of 74 adult chickens of this hatchery were positive in this test.

All of the chicks were kept for 11 days in a common brooder. Starting on the 6th day, they were each weighed daily. In the further course of the experiment, they were weighed every 2 to 8 days. On the 12th day, 26 chicks were infected with 0.2 ml of a 10% yolk sac culture of the chicken strain, 13 birds intramuscularly and 13 intranasally. In the same quarters with these infected birds, 13 other uninfected birds were housed to provide normal exposure. The remaining 40 chicks were kept separate as normal controls.

On the 24th and 25th day after infection, the complement fixation inhibition test was used on the serum of all 79 chicks. The results are recorded in table 1.

TABLE 1.—*Results of the complement fixation inhibition test on the 24th or 25th day after infection.*

Chicken	Serum dilution				
	1:2	1:4	1:8	1:16	1:32
Intramuscularly infected (Petaluma chicken strain)					
58	1+	2+	3+	3+	3+
59	±	±	±	2+	3+
60	±	±	±	1+	1+
61	±	±	−	−	1+
62	±	±	1+	2+	3+
63	1+	2+	2+	3+	3+
64	1+	1+	2+	3+	3+
65	1+	1+	2+	3+	3+
66	±	±	±	1+	2+
67	±	±	1+	2+	3+
68	1+	2+	3+	3+	3+
69	±	±	1+	2+	3+
70	1+	1+	2+	3+	3+
Intranasally infected (Petaluma chicken strain)					
71	1+	1+	1+	2+	2+
72	±	±	±	1+	2+
73	±	±	±	1+	2+
74	1+	2+	2+	3+	3+
75	±	±	1+	2+	3+
76	±	1+	1+	2+	3+
77	1+	2+	2+	3+	3+
78	1+	1+	1+	3+	3+
80	1+	1+	2+	3+	3+
81	1+	1+	2+	3+	3+
87	±	±	1+	2+	3+
94	1+	2+	3+	3+	3+
97	1+	1+	1+	2+	3+
Exposed to experimentally infected birds					
79	3+	3+	3+	3+	3+
83	1+	1+	2+	3+	3+
84	3+	3+	3+	3+	3+
85	±	±	1+	3+	3+
86	1+	1+	2+	3+	3+
88	3+	3+	3+	3+	3+
89	3+	3+	3+	3+	3+
90	±	±	1+	2+	3+
91	1+	1+	1+	3+	3+
92	2+	2+	2+	3+	3+
93	2+	2+	2+	3+	3+
95	±	−	±	1+	3+
96	3+	3+	3+	3+	3+

Fixation control: 4+.

Table 1 shows that all inoculated birds became serologically positive within 25 days after infection, whereas all normal control birds remained negative. Only 6 of 13 normal exposure controls were positive, 2 (92 and 93) were questionable and 5 were negative. The 7 serologically questionable or negative birds were tested 4 more times (table 2).

Accordingly, 2 of 13 exposure controls

TABLE 2.—*Complement fixation inhibition test on chickens exposed to infected birds.*

Chick	Days after exposure									
	40th			53rd		74th			86th	
	1:2	1:8	1:16	1:8	1:16	1:4	1:8	1:16	1:8	1:16
79	2+	4+	4+			4+	4+	4+		
84				4+	4+				±	1+
88				4+	4+	4+	4+	4+		
89	4+	4+	4+			−	±	2+		
92		2+	2+	1+	2+					
93	3+	3+	3+			2+			4+	4+
96				±	1+					

were consistently seronegative 86 days after beginning of exposure (chicks 79 and 88). The 1:2++ reaction of chick 79 after 40 days can hardly be interpreted as positive because it exists only in the dilution of 1:2 and does not persist or rise with further bleedings. One (bird 93) was slightly positive on the 74th day, but again negative at a later date. Four birds became distinctly seropositive after different intervals. Thus, the serologic response of 6 birds was positive by the 25th day; of 1, by the 40th day; of 1, by the 53rd day; of 2, by the 74th day; and 2 remained negative throughout the exposure period.

The presence of infection was reflected in the weight charts. Observation of the weight of the birds was carried through as long as the 52nd day after the beginning of the experiment. There were quite remarkable differences in the individual growth curves within each of the four groups—normal controls, intramuscularly infected, intranasally infected and exposure controls. Therefore, the average weight was determined for each group (fig. 1). Figure 1 shows the highest average weight for the normal control birds; the exposure controls were second; the intranasally instilled, third; the intramuscularly infected birds, fourth. The weight curve of the last group dropped immediately after the day of inoculation. The curve of the intranasally instilled chicks dropped below the normal curve about one week later. The exposure controls, as would be expected, took a few more days to drop below the normal weight average. None of the birds showed signs of grave illness.

On the 65th day after their inoculation, 12 chickens (4 normal exposure controls, 4 intramuscularly injected and 4 intranasally instilled birds) were bled out. Their inhibiting titers are given in table 3 and those found on the 25th day after inoculation are given for comparison.

Table 3 reveals a slight titer increase for some serums, considerable increase

TABLE 3.—*Inhibition titers and virus isolations.*

Chick	Means of infection	Highest serum dilution showing inhibition titers		Virus isolation (65th day)			
		25th day	65th day	Organs	Mouse passage	Cloaca	Mouse passage
58	Intramuscular	1:3+, 1:6++	1:16±, 1:32+	+	First	−	
61		1:24−, 1:48+	1:8−, 1:16+	−		+	First
63		1:2+, 1:4++, 1:8++	1:16±, 1:32+	−		−	
67		1:4±, 1:8+, 1:16++	1:8−, 1:16+, 1:32+	+	First	+	Second
72	Intranasal	1:8+, 1:16+, 1:32++	1:8+, 1:16+	−		−	
75		1:4±, 1:8+, 1:16++	1:32−	−		−	
77		1:3+, 1:6++, 1:12++	1:2 to 1:256++++	−		−	
81		1:4+, 1:8+	1:4−, 1:8+	+	Third	+	First
85	Exposure	1:4±, 1:8+	1:4−, 1:8+	−		−	
90		1:4±, 1:8+, 1:16++	1:8±, 1:16++	−		−	
91		1:8+	1:8+	−		−	
95		1:8±, 1:16+	1:8+, 1:16++	−		−	

for two serums (chicks 58 and 75), considerable decrease in one (chick 61) and loss of titer in two. One bird completely lost its serologic inhibition titer (bird 77) and virus could not be recovered; hence, there is at least a possibility that the bird was not infected. It is interesting that although the inhibition titer of chick 61 decreased, the virus was recovered from cloaca material.

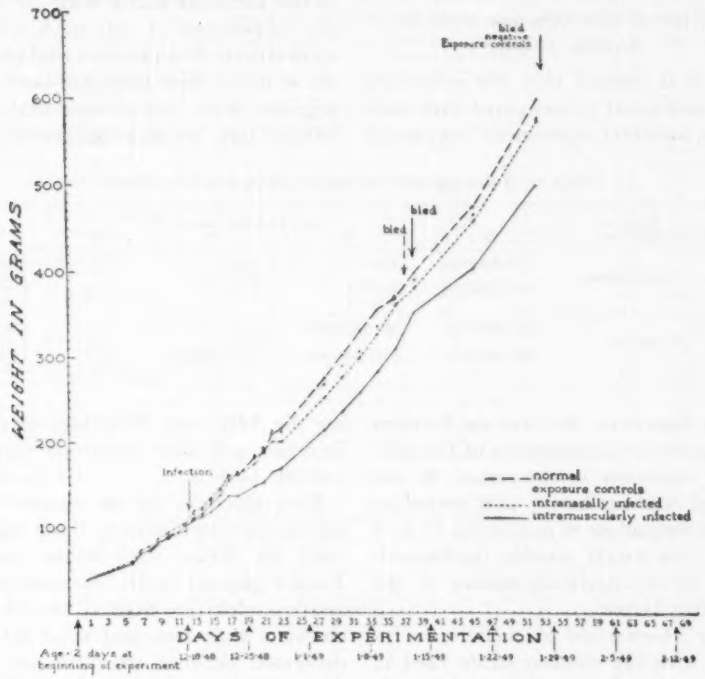

FIG. 1.—Variations in weight in ornithosis-infected chickens.

Gross examination of these 12 birds was negative (gross lesions or enlargement of spleen or liver were absent). Parts of liver, spleen and kidneys were pooled, emulsified in broth and inoculated into white mice intraperitoneally and intranasally. Parts of the cloacas were treated similarly. Negative results of mouse passage does not necessarily indicate that infection is absent; the ornithosis virus is known to be of low infectivity for mice. The positive findings are, of course, significant. The result of this attempt to isolate the virus is also shown in table 3. The isolation of the virus from three intramuscularly injected and one intranasally instilled chicken indicates that intramuscular injection creates a more massive infection than intranasal administration or exposure to infected birds. On the other hand, the virus could not be recovered from 4 birds infected less than 9 weeks, which may evidence the rather fast clearing of the infective agent from the host or the low infectivity of the virus for mice. It is, therefore, not astonishing that in a later experiment the virus could be isolated only from a few serologically positive chickens. From none of the ex-

posed birds was the virus recovered. Hence, further experiments were carried out to evaluate the complement fixation inhibition test because we felt sure that infection was present under the condition of constant exposure.

2. Interval after infection when test becomes positive

Since it seemed that the inhibitory substance could be compared with antibodies, a further experiment was carried out to determine the interval between inoculation and appearance of the inhibitory substance in the serum. If this interval was similar to that necessary for the formation of antibodies (7 to 9 days), this would provide further evidence of the antibody nature of the inhibiting factor.

Four 11-week-old chickens were injected with the chicken strain used in experiment 1. To two birds, 0.5 ml of a 10% yolk sac culture was administered intramuscularly, and approximately 0.4 ml of the same culture was given intranasally to the others. Before inoculation and at different intervals, serum was examined by the complement fixation inhibition test (table 4). It becomes apparent that the intramuscularly infected birds become seropositive earlier than the intranasally instilled ones. With one exception (chicken 43), the serum showed inhibitory action after 7 to 9 days, the same period which is known to be necessary for the production of humoral antibodies. The titers 3 weeks after infection are definitely higher than those observed after 7 to 9 days.

TABLE 4.—*Inhibition titers on experimentally infected chickens.*

Chicken	Route of infection	Days after inoculation 0	7	9	15	21
44	Intramuscular	No inhibition	1:4+++	1:3+ 1:6++		1:16+ 1:32++
51		No inhibition	1:3++ 1:6++	1:2++		1:16± 1:32+
10	Intranasal	No inhibition	No inhibition	1:2++ 1:4++		1:8± 1:16++
43		No inhibition	No inhibition	No inhibition	1:6+ 1:12+	1:32± or higher

3. Strain specificity

Seventeen white Leghorn chickens, approximately 7 weeks old, were injected intramuscularly with 20% yolk sac suspensions (1 ml) of 8 different virus strains. In a previous trial bleeding the serum of these birds had been found negative in the complement fixation inhibition test. Serum samples were taken on the 12th and 20th days after the injection and their inhibition titers recorded (table 5).

Four chickens did not respond with significant titers, namely those injected with the Baker (cat) strain and the Endo 3 (pigeon) strain. The complement fixation inhibition titers of the 13 other chickens were low, and most of them decreased between the 12th and 20th day; 2 (chickens 98 and 89) turned practically negative after having been positive on the 12th day. Only 3 serums showed increased inhibition between the 12th and 20th day (chickens 90, 91, 97). There seemed to be a certain relationship between inhibition titers and infecting strains, insofar as a few strains produced somewhat higher inhibition titers after one injection than others, especially the Petaluma duck, the Seattle parakeet, and the Louisiana strains, whereas the cat virus and the Endo 3 strain were not able to produce the inhibitory substance after one inoculation.

In one further experiment, normal beef serum was used as antigen for immunization of adult chickens. The cooperation of Dr. H. R. Wolfe, Associate Professor of Zoology, University of Wisconsin, made it possible to obtain two more antibeef chicken serums with precipitation titers. Chickens were given 5 to 15 intravenous injections of beef serum; the complement-fixing serum was obtained from an immunized rabbit. The precipitation titers in our birds did not reach the height of those described by others (Wolfe, 1942; Wolfe and Dilks, 1946, 1948, 1949). Chicken serums with precipitin titers not lower than 1:100 were tested by the complement fixation and the complement fixation inhibition method. Four of 8 chicken serums inhibited complement, but none of the 8 serums fixed complement (table 6).

TABLE 5.—*Results of the complement fixation inhibition test with various strains of the psittacosis virus.*

Chicken	Strain and source	Days after inoculation									
		12					20				
		1:2	1:4	1:8	1:16	1:32	1:2	1:4	1:8	1:16	1:32
83	Petaluma (duck)	−	−	−	1+	3+	−	−	3+	4+	4+
85		−	−	±	2−	4+	−	2+	4+	4+	4+
82	Baker (cat)	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+
95		4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
90	Lymphogranuloma	±	1+	2+	4+	4+	−	±	1+	3+	4+
98	venereum	±	±	1+	4+	4+	3+	4+	4+	4+	4+
89	Bar Harbor	±	3+	4+	4+	4+	3+	4+	4+	4+	4+
91	(mouse)	1+	1+	2+	4+	4+	−	−	2+	4+	4+
84	Seattle (parakeet)	−	−	−	1+	3+	−	1+	4+	4+	4+
86		−	1+	3+	4+	4+	1+	3+	4+	4+	4+
97	Louisiana	1+	2+	3+	4+	4+	−	−	±	3+	4+
101	(human)	−	−	−	1+	3+	−	−	±	3+	4+
88	Endo 3 (pigeon)	3+	3+	4+	4+	4+	3+	4+	4+	4+	4+
93		3+	4+	4+	4+	4+	4+	4+	4+	4+	4+
102	Petaluma "B"	3+	±	−	1+	4+	−	−	±	2+	3+
103	(chicken)	1+	1+	3+	4+	4+	1+	3+	4+	4+	4+
104		1+	2+	4+	4+	4+	1+	3+	4+	4+	4+

Fixation control: 4+.

TABLE 6.—*Results of test with antibeef chicken serum.*

Chicken	Titers						
	Precipitin			Complement fixation inhibition*			
	1:100	1:1000	1:10,000	1:4	1:8	1:16	1:32
I(2–23)	3+	2+	−	−	±	1+	
y 6031	3+	1+	±	−	−	1+	2+
L	1+	±		1+	2+	3+	4+
PC100†	3+	2+	2+		±	1+	3+
Brown	2+	1+	1+	4+	4+	4+	4+
I(4–15)	2+	1+			4+	4+	4+
M	1+			4+	4+	4+	4+
PC103†	2+	1+	1+	4+	4+	4+	4+

* Fixation control: 4+.
† Obtained through the courtesy of Dr. H. R. Wolfe.

4. Application of the test

On the basis of all the experiments previously reported, it was logical to assume that a positive complement fixation inhibition test is indicative of ornithotic infections in chickens. Consequently, the test was used on a small

scale to determine the presence of infection at several farms and hatcheries and in other instances in which an opportunity was afforded. The age of most birds was between six months and two years; they were of different breeds. The results are given in table 7. Serums with very slight inhibition in the lowest dilution only are considered questionable. The titers were all rather low, with only one exception in which complete inhibition was observed in a dilution of 1:64. In many of the tests, inhibition was only partial even in the low serum dilutions, but inhibition titers observed in 26 inoculated baby chicks (experiment 1) were not higher than those in the field specimens, and quite a few of the inoculated also showed only partial inhibition. These low titers in the field specimens must, therefore, be considered as specific. If the chicken serum had been incubated with the antigen before other reagents were added, the inhibition titers obtained might have been slightly higher (Karrer et al, 1950). Instead, the "standard" method (Karrer et al, 1950) was employed throughout the survey because of its greater simplicity.

In a few instances the highest inhibition titers were not observed in the lowest dilutions. This is due to anticomplementary action of the serum in low dilutions, which is expressed by incomplete hemolysis in the serum control tubes.

The low incidence of positive inhibi-

TABLE 7.—*Results of application of the complement fixation inhibition test.*

Source	Total birds tested	Positive		Questionable	Negative	Remarks
		No.	Percent			
Chickens						
Hatchery: California	74	4	5.4		70	
Smalls farms:	37	28	75.7	7	2	
	20			8	12	
California	9	5	55.6	3	1	Pigeon-breeding farm
	1				1	Bird had ocular lymphomatosis
	20	10	50.0	1	9	Owner had ornithosis (Karrer et al, 1950)
Laboratories: California	3				3	Birds used for other experiments; high Newcastle virus hemagglutination inhibition titers
Michigan	69	4	5.8		65	Regional Poultry Lab., East Lansing
California	9	2	22.2		7	Dept. of Agriculture, Sacramento
Iowa	15			2	13	Iowa State College, Ames
California	12	12	100.0			Unhealthy when purchased from breeding ranch
Totals	269	65	24.2	21	183	
Ducks						
Duck farms: California	39	23	59.0	3	13	Dirty farm; ornithosis virus isolated from birds 2 years before
Long Island	65	32	49.2	10	23	Serum stored in refrigerator for 3 years; virus isolated from 15 of 65
	(15)	(8)	(53.3)	(4)	(3)	Results for above 15
Totals	104	55	52.9	13	36	
Crow						
Nature: California	1				1	
Turkeys						
Laboratories: Colorado	3				3	Colo. A. & M., Fort Collins. Birds had sinusitis.
California	45	1	2.1	7	37	Dept. of Agriculture, Sacramento; serum of 3–5 birds pooled; many serums contaminated and anticomplementary
Totals	48	1	2.1	7	40	

tion reactions in the tested turkey serums compared with the chicken and duck samples, has to be interpreted with care. Since practically all turkey samples consisted of pooled serum from 3 to 5 birds, this dilution undoubtedly colored the results. Weakly inhibiting serum would not be detected. The 7 turkey samples which gave questionable results probably did contain a positive component which could not show up except in the lowest dilution. Therefore, the incidence of ornithosis among the tested turkeys can be assumed to be higher than the 2.1% found (table 7) with the described technique. Because inhibition is mostly evident in low titers only, it is imperative that samples be tested individually, not pooled, in order not to miss most of the positive reactors.

Although in an earlier study the virus was isolated only from 4 of 8 chickens 9 weeks after their experimental infection (table 3), further attempts were made with the organs of 56 chickens and ducks, most of which were serologically positive. They were killed soon after purchase, and mice were inoculated intranasally and intraperitoneally with emulsions of organs from 38 of these birds. Parts of liver and spleen were pooled and inoculated; parts of both kidneys were generally injected separately, but sometimes they were pooled with liver and spleen. Ground-up cloacal wall and contents were not pooled with the organs. They were always injected separately into mice. Three successive mouse passages were carried out. Only in a few instances in which the organs of the mice suggested but did not definitely confirm the presence of the virus on the third passage was a 4th or even 5th passage carried out. The lungs of the intranasally inoculated mice were passed only intranasally, liver and spleen of the intraperitoneally infected animals only intraperitoneally. Mice of this strain had been used at the Hooper Foundation for nearly 10 years for psittacosis work. They had been tested periodically for mouse pneumonitis virus by one of us (B. Eddie), and in these years it had never been recovered from the lungs of the mice. The animals were therefore considered to be free of this virus and hence suitable for intranasal passage.

A psittacosis-like virus was isolated from six birds. It was generally recovered only on the third mouse passage and did not kill the animals. One strain was adapted to the yolk sac of the chick embryo and used in experiment 1. The yolk sac cultures were very highly infective for mice, when injected either intraperitoneally or intranasally.

5. Reaction of pigeon serum

The complement fixation test has been useful in the testing of pigeon serum ever since Meyer (1941) and Meyer, Eddie and Yanamura (1942) proved its value for the detection of antibodies in pigeons. On numerous occasions, however, when birds were injected with psittacosis virus, demonstrable complement-fixing serum antibodies did not necessarily develop, although in other pigeons, considerable serum antibody levels appeared after one or two injections of virus. This difference in the immunologic behavior was explained by the assumption that the birds that made no serologic response had had previous contact with the virus after which complement-fixing antibodies had developed and later disappeared. Since pigeons actually may be carriers of psittacosis-like viruses (Coles, 1940; Meyer, 1941), it can easily be assumed that the serum of such birds does not react to injection of virus once it has lost its measurable antibody titers.

In an attempt to get such complement-fixing pigeon serums for use in complement fixation inhibition tests, 12

birds were purchased from a loft that had been proved to be heavily contaminated with the virus. Old birds, 5 to 7 years of age, were chosen in the belief that they all would have had ample chance to come in contact with the virus during their long life. The birds were bled out and their serum was tested by the complement fixation method. Serum which did not show high titers was examined by the complement fixation inhibition test. Results of both tests are shown in table 8.

TABLE 8.—*Complement fixation and complement fixation inhibition with pigeon serum.*

Pigeon	Tests*	Serum dilution									
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
1a	CF	1+	2+	1+	–	–	–	–	–		
	CFI	1+	1+	2+	3+	3+	3+	4+	4+	4+	4+
2	CF	4+	4+	4+	4+	4+	4+	4+	4+		
1	CF	–	–	–	–	–	–	–	–		
	CFI	–	–	–	–	–	–	–	–	–	±
5	CF	–	–	–	–	–	–	–	–		
	CFI	–	–	–	–	–	±	1+	2+	3+	3+
6	CF	4+	4+	4+	4+	4+	4+	1+	–		
8	CF	–	–	–	–	–	–	–	–		
	CFI	–	–	–	–	±	±	1+	1+	1+	2+
9	CF	4+	4+	4+	4+	4+	4+	3+	±		
11	CF	–	–	–	–	–	–	–	–		
	CFI	±	±	–	±	–	±	±	±	1+	2+
13	CF	±	1+	3+	4+	3+	2+	–	–		
	CFI	±	±	1+	2+	3+	4+	4+	4+	4+	4+
40	CF	4+	4+	4+	4+	4+	4+	3+	–		
45	CF	–	–	–	–	–	–	–	–		
	CFI	–	–	–	–	–	–	±	±	1+	2+
66	CF	–	–	–	–	–	–	–	–		
	CFI	±	±	1+	1+	2+	2+	3+	3+	4+	4+

Fixation control: 4+.
* Complement fixation test with approximately 4 units of antigen; complement fixation inhibition test with approximately 1 unit of antigen.

Table 8 plainly shows the extremely interesting fact that all serums giving negative results in the complement fixation test markedly inhibited complement fixation if tested by the complement fixation inhibition test. The observed inhibition titers were, for the most part, considerably higher than those observed with chicken serum. One sample inhibited complement fixation even if diluted as high as 1:1024. Not a single serum was negative in both tests.

Serum 13, which showed a "prozone" phenomenon in the straight complement fixation test, inhibited complement fixation in those low dilutions in which it did not, or only partially did, fix complement. This serum was therefore positive in both tests. Serum 1 also showed slight titers in both tests. This observation—that one serum can combine with antigen both with and without the fixation of complement—strongly supports our belief that the inhibiting substance is an antibody (see Discussion).

Several months later serum 13 was retested. The procedure for the complement fixation and complement fixation inhibition tests was modified in incubation and in quantity of antigen in order to determine if the complement fixing antibody could be demonstrated also in the lowest serum dilutions in which the serum showed a "prozone" phenomenon in the complement fixation test and inhibition in the complement fixation inhibition test. Results are given in table 9. This experiment revealed very interesting facts. A comparison of series "b" and "d" shows that serum 13 inhibits complement fixation in the dilutions in which it fixes complement only partially. If, however, 4 more units of antigen are added and the mixture is incubated, full fixation is observed also with the lowest serum dilutions. This seems to prove the assumption that some substance in serum 13 interferes with the fixation of complement, causing "prozone" phenomenon and inhibi-

tion, respectively, in complement fixation and complement fixation inhibition tests. After this substance has been neutralized by 1 unit of antigen, complement fixation is made possible if 4 more units of antigen are added. However, series "f" shows that serum 13 does fix complement also in the low dilutions if more antigen is added from the beginning. This contrasts with previous findings (table 8) that serum 13 gave the typical "prozone" also in presence of 4 units of antigen, whereas, in the second experiment (table 9) it only did so in the presence of 1 unit. This discrepancy is probably due to changes in the pigeon serum during a storage of several months and perhaps also to the fact that a different batch of antigen was used in the later experiment. The "prozone" reaction in series "d" cannot be considered to be due to lack of antigen (only 1 unit) or serum excess, because this would not explain the inhibition power of serum 13 in the presence of this same amount of antigen plus serum 9. The reported results are much easier to explain if it is postulated that some substance in serum 13 inhibits the complement fixation by both antibodies, the one of serum 9 and the other of serum 13 itself. It was impossible to plan systematic experiments to observe the behavior of pigeon serum in the complement fixation and the complement fixation inhibition tests during a period of artificial immunization with psittacosis virus in order to study this further.

6. Reaction of human serum

After it had become obvious that pigeons could be serologically positive in either complement fixation or complement fixation inhibition tests, the question arose if this was the case also with human serum. Since Bedson (1933 and 1935) had demonstrated the value of the complement fixation technique for convalescent serum, this test has been the most reliable laboratory method in the diagnosis of human psittacosis except isolation of the virus from blood or sputum. Also, since complement-fixing antibodies can be demonstrated in man in a great many other infections, the antibody in human serum seems to have little in common with the inhibiting substance of fowl serum. Nevertheless, one human serum that had shown a prozone phenomenon in the complement fixation test showed incomplete inhibition if tested by the complement fixation inhibition method (table 10). Three more specimens, obtained during or after the patient had been treated with aureomycin, were all negative by the complement fixation method. Two were tested for inhibitive antibodies and found negative. In the test of March 4, the serum had shown a prozone phenomenon in the straight complement fixation test, which was a probable indication for inhibitive properties. The serum could not be tested by the complement fixation inhibition method until several weeks later, and at that time it did not show any complement fixation.

TABLE 9.—*Results of complement fixation and complement fixation inhibition tests with a serum positive in both tests.*

Tube series*	Pigeon serum	Antigen unit	Preliminary incubation	Antigen units	Pigeon serum	Complement units	Incubation	Results				
								1:4	1:8	1:16	1:32	1:64
af	13	1	2 hr., 37 C	4		2	2 hr., 37 C	4+	4+	4+	4+	4+
b	13	1	2 hr., 37 C		9	2	2 hr., 37 C	±	±	1+	3+	4+
c	13	1	None		9	2	2 hr., 37 C	±	1+	1+	3+	4+
d	13	1	2 hr., 37 C			2	2 hr., 37 C	−	±	1+	2+	3+
e	13	1	None			2	2 hr., 37 C	1+	1+	2+		
f	13		None	4		2	2 hr., 37 C	4+	4+	4+	4+	3+

* b and c—CFI test; a, d, e f—CF test.
† Absorption of inhibiting substance by preliminary incubation with 1 unit of antigen.

This loss of complement-fixing titer was probably due to storage in the refrigerator and repeated inactivation at 56 C. Nevertheless, fixation was partially inhibited in two tests in the tubes containing the low serum dilutions, and this inhibition corresponded to the prozone phenomenon previously noted. The behavior of this serum in the complement fixation and complement fixation inhibition tests is similar to that of a pigeon serum (pigeon 13) as described above (tables 8 and 9), although it could not be proved that the human serum possessed complement-fixing and inhibiting properties at the same time in the same test.

TABLE 10.—*Complement fixation inhibition test of human serum exhibiting a prozone reaction in the complement fixation test.*

Date, 1949	Test	Titer						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
March 4	CF		2+	3+	3+	3+	3+	2+
April 27	CFI	2+	1+	2+	2+		4+	4+
	CF	–	–	–	–	–	–	–
May 25	CFI		1+	1+	3+		4+	4+
	CF	–	–	–	–	–	–	–

DISCUSSION

The reported experiments definitely prove that inhibition titers can be induced in chickens through intraperitoneal or intranasal administration of different psittacosis-like viruses, and that noninfected birds may acquire inhibition titers if they are housed with freshly infected ones. Titers are demonstrable 7 to 9 days after injection of virus and often rise in the following days. All these facts speak strongly in favor of the antibody nature of the inhibiting substance in fowl serum, which is produced when the bird becomes infected. This idea was presented by Rice (1948), who found inhibition titers with serum of *Salmonella pullorum*-infected chickens which ran more or less parallel with the agglutination titers.

The serologic picture did not indicate the clinical severity of the infection in the experimental work reported herein. Whereas the only objective signs of illness could be measured by a slight retardation of growth, the inhibition titers produced by one single injection of chicken virus were still present in 11 of 12 birds 9 weeks afterward. The infection in chickens is practically subclinical. None of 12 sacrificed birds showed gross anatomical lesions, and the virus could be recovered from only 4 of them. Either the virus disappeared rapidly from the host, or it was not highly infective for the white mice used in these isolation attempts. Probably both factors are of importance. The fact that 3 of 4 intramuscularly infected chickens still harbored the virus after 9 weeks, but none of 4 exposure control birds did, at least suggests that the concentration of the virus is a factor, because the intramuscular route is apt to produce a higher concentration of virus than intranasal instillation or simple exposure. The high infectivity of the chicken strain yolk sac culture for mice also speaks against the hypothesis that chicken strains are of little virulence for this laboratory animal. Although the virus is so difficult to recover a short time after experimental injection of chickens, it cannot be assumed that it has disappeared completely from the host. Apparently healthy chickens can even become the source of human infections (Karrer, Eddie and Schmid, 1950).

The weight curves of infected chicks as determined during a period of several

weeks show most retardation of growth for intramuscularly infected birds, the least for normal exposure controls. This again indicates the much milder character of intranasal infection or infection by exposure than that induced by injection of virus. The gradual retardation of growth observed in the normal exposure controls illustrates graphically, so to speak, the gradual infection of this group, which is also reflected in the results of complement fixation inhibition tests with serum samples obtained at different intervals.

Quite different strains of virus are apt to induce inhibition titers in chickens, and the reaction does not seem to be particularly strain-specific in ornithosis. This is not unexpected because strain-specific psittacosis antigens are not available. The Endo pigeon strain and Baker's cat strain obviously appeared less apt to induce antibody formation in chickens than the six other strains of psittacosis-like viruses. One injection of these strains did not invoke inhibition titers.

The high percentage of positive results with field specimens is striking. Virus isolation attempts were not more successful in this group than in the experimentally infected chickens. The field surveys do indicate that the infection rate among barnyard fowl may be high, as it is among pigeons (Coles, 1940; Meyer, 1941; Meyer, Eddie and Yanamura, 1942). The high incidence of seropositive reactions among the birds makes it imperative to test chickens to be used in psittacosis experiments, in order to rule out the naturally infected carriers.

The results obtained with pigeon serum and one human serum further confirm the antibody nature of the inhibiting substance. Assuming that the inhibiting substance is an antibody, this antibody, which does not fix complement but combines with antigen, must be different from the complement-fixing antibody because it is found independent from the other in serum of the same animal species. Rarely both antibodies occur in the same serum. This is to be suspected in all cases of a prozone phenomenon in the straight complement fixation test. This is the first observation of this kind. It probably will be found in other instances in which prozone phenomena occur. Rice (1948) mentioned the prozone effects observed in the course of immunization with pneumococci (horses) and *Salmonella pullorum* (chickens), which might well be due to the presence of the inhibitive antibody that masks the complement-fixing antibody in the lower dilutions. This latter might be demonstrated also in the low dilutions by using more antigen or by employing an absorption technique, as used for pigeon serum 13 (table 9). Nevertheless, the number of samples containing both antibodies (one human, one pigeon serum) is too small to allow far-reaching conclusions.

The type of antibody obviously depends on the animal species. Chickens form nearly exclusively the inhibiting and humans and guinea pigs the complement-fixing antibody. Pigeon serum has an intermediate position between those two extremes, a fact which is probably responsible for their usefulness in the inhibition test, because they do not fix complement very firmly and fixation can be easily inhibited by fowl serum. The significance of either inhibiting or complement-fixing antibody in the clinical picture must be further investigated. There is a possibility that human serum negative by the complement fixation test might be found positive by the inhibition method, perhaps for some time after the disease, when an initial complement fixation titer has disappeared. The complement fixation inhibition test would, in this case, be a supplementary test, to be run especially with

serum samples negative in the complement fixation test.

SUMMARY

The complement fixation inhibition test has been applied to the serum of 26 ornithosis-infected and 13 normal chickens (exposed to the infected ones), with positive outcome except for two serums. The results with serums of the same number of normal birds were negative. Weight curves of the infected and exposed groups showed retardation of growth, compared with the normal group. Inhibition titers persisted for at least 9 weeks, after which the virus could be recovered from only 4 of 12 birds.

Inhibition titers were found not earlier than 7 days after injection or intranasal instillation of virus. From then on to the 20th day sometimes a rise in titer occurred and sometimes a reduction. Of 8 virus strains, 6 were able to produce inhibiting antibody after one injection. There was no marked correlation between strain and height of titer in the titer-producing group.

In a survey of 269 chicken serums, 24.2% were positive to the inhibition test; of 104 duck serums, 52.9% were positive; and of 48, mostly pooled, turkey serums, 2.1% were positive. Isolation attempts using organs and cloacas of 38 chickens and ducks were successful in 6 instances.

Pigeon serum either fixed complement or inhibited fixation. One pigeon and one human serum were found positive in both tests. This observation may be of some significance in the serologic investigation of human specimens.

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THE DYNAMICS OF HEMOLYSIN FORMATION IN INTACT AND SPLENECTOMIZED RABBITS

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The rise and fall of hemolysins have been studied in intact and splenectomized rabbits following a single standardized intravenous injection of sheep red cells. We selected hemolysin production for investigation because antibody activity can be rapidly and quantitatively determined by photometric methods at frequent intervals in the same animal. A comparison of the serum antibody curves in intact and splenectomized animals indicates strongly that the spleen in intact animals usually forms a large part of the antibody which accumulates to give the initial peak serum titer, and then abruptly ceases to form antibody. After the peak titer, therefore, spleen-produced antibody in the serum decays at a rate approximately characteristic of the half life of antibody until the titer reaches the approximate level characteristic of nonsplenic sources. Nonsplenic sources maintain a low level of serum antibody over long periods. This work has been briefly reported by us (1950a and b).

Burnet and Fenner (1949) have critically reviewed the literature on the rise and fall of antibodies after various types of immunization. We know of no work on hemolysins in rabbits which has utilized the accuracy of the 50% photometric titration and which was carried out over long periods at frequent intervals.

It is well established that extirpation of the spleen at about the time of immunization reduces acquired immunity including the formation of specific antibodies in some animals. A decreased production of hemolysins as a result of splenectomy has been reported in the dog by Hektoen (1909) and Luckhardt and Becht (1911), in the rabbit by Motohashi (1922) and by Scarff (1931) if done 1 to 6 hours after immunization, and in the rat by Hektoen (1920) and Rowley (1950). The work of the last investigator indicates that the effect of splenectomy is limited to the intravenous route of immunization with small doses of antigen.

MATERIALS AND METHODS

Rabbits are numbered in chronological sequence. Rabbits 1 to 9 were tested in 1947, rabbits 14 to 22 in 1948, rabbits 28 to 60 in 1949 and rabbits 70 to 113 in 1949–50. The first batch was snuffles-free. All others were obtained from one source and were in good condition. They gained weight regularly. They were watered and fed daily with Dixie rabbit pellets from the Dixie Mills Company, East St. Louis, Illinois, and were given carrots twice a week. They were maintained on an automatic 12-hour light and dark schedule. They usually weighed from 2 to 3.2 kg at the time of immunization.

All rabbits received a single immunizing dose of sheep red blood cells diluted in ordinary physiological saline (0.85% sodium chloride) and injected into a marginal ear vein. Blood was drawn sterilely and defibrinated 1 to 6 days before injection. The cells were washed in an excess (diluted about 1:100) of physiological saline by centrifuging for 6 minutes on the day they were injected. They were resuspended in physiological saline and standardized on the Klett-Summerson (cf. Summerson, 1939) photoelectric colorimeter with a 54 filter (approximately 500–570 mμ).

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This colorimeter was used throughout the work for standardizing all red cell suspensions either for injections or for in vitro tests (see below for details). Red cells from the same sheep were used for all in vitro tests and for the immunization of rabbits 1 through 9. Red cells from 1 to 4 other sheep were used for injections into rabbits in subsequent experiments.

Three different immunizing injections were given. The smallest one consisted of 1.25 ml of 0.1% sheep cells standardized so that 1 ml plus 4 ml distilled water gave a reading of 150 on the colorimeter (equivalent to about 1% packed cells). The intermediate one consisted of 1.25 ml of 1% sheep cells standardized as was the small dose. The large one was prepared as follows: A suspension of red cells was standardized so that 1 ml plus 4 ml distilled water gave a reading of 300 on the colorimeter. This suspension was then centrifuged and four-fifths of the supernatant was removed. One ml of the remainder (equivalent to 10% packed red cells) constituted the dose. All antigen injections were given per kg rabbit and in approximately the same volume of liquid. For convenience, these standardized suspensions of red cells will be referred to briefly as 1.25 ml 0.1% or 2×10^7 cells, 1.25 ml 1% or 2×10^8 cells and 1 ml 10% or 1.6×10^9 cells, respectively.

Blood for all tests was obtained from one sheep at various times. It was drawn into Alsever's solution as modified by Kent et al (1946). The resulting mixture for convenience was immediately redistributed into test tubes in approximately 7 ml amounts. The tubes were then closed with rubber stoppers to prevent evaporation and were kept in a refrigerator at 6 C. Samples were tested for sterility.

A hemolysin unit is defined as the smallest amount of serum which produces 50% hemolysis of 1 ml of a standardized suspension of sheep red cells in the presence of four 50% units of complement under the conditions outlined below. Titers are expressed in two ways in the present paper. Actual amounts of serum, representing the titers as defined above, are indicated in the graphs. In the text and in the tables, titers are given as a dilution of antiserum in 1 ml. Such dilutions are obviously the reciprocals of the titers given in the figures. Hence, the minus logarithms of these dilutions can be used in the von Krogh equation.

All titrations are based on von Krogh's (1916) equation:

$$x = K\left(\frac{y}{1-y}\right)^{1/n}$$

in which x is the amount of hemolytic serum (or of complement when complement is being titrated); y is the degree of hemolysis expressed as a fraction of one, and K and $1/n$ are constants of which K is the 50% unit of hemolysin (or of complement). After logarithmic transformation, a plot of $\log x$ and $\log (y/1-y)$ will give a straight line with a slope of $1/n$ and the log of the 50% unit can be read from this plot. For the advantages of this method over determinations of 100% hemolysis, the reader is referred to Wadsworth et al (1931).

The actual procedure for titrating a given series of serums involved the following: Rabbits were bled from a marginal ear vein into small tubes. Serums were removed from rabbit blood on the same day or the day after bleeding and were stored in a refrigerator (6 to 10 C). They were inactivated at 55 to 56 C for 20 minutes. They were usually tested within one to three days and were diluted as desired on the day of the test. Occasionally, they were diluted on the preceding day since diluted serums kept overnight in the refrigerator appeared not to diminish in titer.

A veronal buffered saline with added calcium and magnesium ions, according to the formula advocated by Mayer et al (1946 and 1947), was used for all manipulations and dilutions except for injections into rabbits. This buffered saline will simply be designated as saline hereafter.

On the day of the test, sheep red cells were washed in about 50 times their volume of saline and were standardized, i.e., 1 ml when added to 4 ml distilled water gave a reading of 300 in the colorimeter. At first, the red cells and distilled water were centrifuged before standardizing. Later, this centrifugation was found to be unnecessary.

Complement was titrated with standardized red cells and hemolysin of known titer according to table 1. Illustrative readings of different batches of complement may be found in the table. By graphing the logs of the dilutions against the logs of $y/1-y$ in which y equals the percent hemolysis, the 50% unit of a particular batch of complement was obtained and a dilution made such that four 50% units were contained in 1 ml (table 1). Four 50% units of the lyophilized complement we used fell most often within a range of 0.018 to 0.02 ml undiluted serum or a dilution of 1:50 to 1:55. Lyophilized complement was obtained from Markham Laboratories, Chicago, Illinois and Carworth Farms, Inc., New City, New York.

Each antiserum was set up in a series of regularly spaced dilutions with respect to log values in Wassermann tubes. Four to 6 dilutions of antiserum were generally used with the hope that at least 3 or 4 would fall between the 20 and 80% values of hemolysis. To determine the particular

dilutions of a given serum, the previous titer was used as an orientation point, and dilutions below and above this point were made. Ordinarily, for example, when the previous titer was 1:800 (log −2.9), the log of the 6 dilutions for the current test would begin with log −2.6 and descend by 0.1 steps to −3.1. From a complete table, such as the sample shown in table 2, it could then be easily determined whether a basic dilution of 1:10, 1:100 or 1:1000 serum should be made and how much of the particular dilutions of serum and of saline were needed for the 6 tubes in the current test. When the titers of a set of serums were rapidly rising, a preliminary 3-tube test with 0.4 to 0.7 log steps oriented the range for the final test. When weakly hemolytic normal serums were being titrated, 9 or more tubes had to be set up.

The standardized sheep cell suspension was then added to each tube. The tubes were gently shaken by inverting two or three times and were reassembled into sets of 27 per rack. After 10

TABLE 1.—*Protocol to obtain the 50% unit of complement and results on three samples of complement.*

Tube		1	2	3	4	5	Resultant titer (one 50% unit)		Dilution of complement to contain 4 50% units in 1 ml
							Dilution in 1 ml	Undiluted	
Ml hemolysin 1:500		1	1	1	1	1			
Ml sheep rbc*		1	1	1	1	1			
Ml Saline		2.7	2.6	2.5	2.4	2.2			
Tubes shaken gently and left standing at room temperature for 10 minutes.									
Complement Dil. in ml (=x)		1:333	1:250	1:200	1:167	1:125			
Log of x		−2.52	−2.4	−2.3	−2.22	−2.1			
Ml 1:100 dilution		0.3	0.4	0.5	0.6	0.8			
Tubes shaken, covered and incubated at 37 C for 30 minutes in water bath. Tubes centrifuged at 1500 r.p.m. for 6 minutes. Supernatant from tubes poured into colorimeter tubes and read in the colorimeter.									
Readings of three different samples of complement	1	24	78	113	158	208	1:172	.0058	1:43
	2	27	107	160	202	244	1:212	.0047	1:53
	3	97	163	210	234	282	1:260	.0038	1:65

* Standardized photometrically as described in text.

TABLE 2.—*A portion of the table used to obtain the dilution of serum, amount of serum and the amount of saline needed for hemolysin titrations.*

Hemolysin dilutions in 1 ml	Log of amount of serum	Serum 1:10	Saline*	Serum 1:100	Saline*	Serum 1:1000	Saline*
1:200	−2.3	.05	2.95	.5	2.5		
1.250	−2.4	.04	2.96	.4	2.6		
1.333	−2.52	.03	2.97	.3	2.7	3	0
1:400	−2.6	.025	2.975	.25	2.75	2.5	0.5
1:500	−2.7	.02	2.98	.2	2.8	2.0	1.0
1.666	−2.82	.015	2.985	.15	2.85	1.5	1.5
1:800	−2.9	.0125	2.9875	.125	2.875	1.25	1.75
1:1000	−3.0	.01	2.99	.1	2.9	1.0	2.0
1:1250	−3.1			.08	2.92	.8	2.2
1:1670	−3.22			.06	2.94	.6	2.4

* To facilitate accuracy in pipetting the red cells into each tube, only the fraction of the ml of saline desired was usually pipetted into a tube wherever more than 1 ml was needed. The balance was added with the red cells, i.e., 3 ml of a 1:3 dilution of the standardized red cell suspension.

TABLE 3.—*Protocol to obtain the 50% unit of hemolysin* for antiserum from a rabbit 10 days after immunization with sheep red cells.*

Tube		1	2	3	4	5	6
Antiserum	Dilution in 1 ml (=x)	1:2000	1:2500	1:3333	1:4000	1:5000	1:6666
	Log of x	−3.3	−3.4	−3.52	−3.6	−3.7	−3.82
	Ml of 1:1000 dilution	0.5	0.4	0.3	0.25	0.2	0.15
Ml of saline†		2.5	2.6	2.7	2.75	2.8	2.85
Ml sheep red cells‡		1.0	1.0	1.0	1.0	1.0	1.0
Tubes shaken gently and left standing at room temperature for 10 minutes.							
Complement: 4 50% units in 1 ml		1.0	1.0	1.0	1.0	1.0	1.0
Tubes shaken gently, covered and incubated at 37 C for 30 minutes in water bath. Tubes centrifuged at 1500 r.p.m. for 6 minutes. Supernatant from tubes poured into colorimeter tubes and read in the colorimeter.							
Reading		197	170	134	101	65	45
Percent hemolysis (=y)		66	57	45	34	22	15
Log $y/1-y$§		+.29	+.12	−.09	−.29	−.55	−.75

* With each daily series two control tubes were set up containing (1) complement and sheep cells in saline and (2) sheep cells in saline. In addition, antiserum control tubes were set up when the dilution of antiserum per ml was less than 1:10 and showed some hemolysis. The value obtained was subtracted from the value for the corresponding test.

† Saline was usually added before the antiserum.

‡ Standardized photometrically as described in text.

§ These values were obtained from a table such as table 4 and were plotted against the log of the serum dilution to obtain the 50% titer of the hemolysin.

minutes, four 50% units of complement were added to two racks of 27 tubes at a time and were treated as indicated in table 3. Complement was kept cold until added to the tubes. A schedule was arranged whereby red cells were added every 10 or 15 minutes to batches of 54 tubes. This number was found convenient because the two Servall angle centrifuges we used each had a capacity of 27 Wassermann tubes. Since the metal centrifuge tubes were numbered, only the first tube in the titration of a given antiserum had to be labelled. The supernatant fluid from each tube was poured into matched colorimeter tubes and read in the colorimeter within a half hour. The tubes were calibrated to read within three points near the 150 point. Two sets of colorimeter tubes were used alternately for several serums by inverting and draining one set over several layers of cheese cloth in a rack while using the other set. The colorimeter reading, which was three times the percent hemolysis, was transformed to a log value. For this, a table was constructed of which a portion is submitted in table 4. Logs of the serum dilutions were then plotted against logs of $y/1-y$ in which y is the percent of hemolysis. After fitting a line by inspection, the logarithm for the 50% value of x was read directly and was converted into hemolysin titer from a table, of which table 5 shows a portion.

With some serums (especially those before immunization, during a sudden rise in titer, or from aberrantly reacting rabbits) another test had to be set up because the dilutions of the current test failed to be near the 50% hemolysis point. Such retests were run that afternoon or within the following few days. One serum, when tested 24 times within 4 days by the three workers who titrated the serums, gave a mean of $1:442 \pm 4.3$.

The most satisfactory curves of hemolysin during two months after immunization were obtained from daily bleedings from the 2nd to the 10th day, three times a week for two weeks and twice weekly thereafter. In addition, 12-hourly bleedings from the 4th to 10th day were often made.

TABLE 4.—*A portion of the table used to obtain* log $y/1-y$ *for the percent hemolysis (also readings on the colorimeter) for the 50% unit of hemolysin.*

Colorimeter reading	Percent hemolysis		Log $y/1-y$		Percent hemolysis	Colorimeter reading
137–39	46	*Minus log* ↑	.07	↑ *Plus log*	54	161–63
140–42	47		.05		53	158–60
143–45	48		.04		52	155–57
146–48	49		.03		51	152–54
149–51	50		0		50	149–51

TABLE 5.—*A portion of the log table used to convert the log of the titer into titer expressed as a dilution in 1 ml.*

Characteristic	−1	−2	−3
Mantissa of negative log			
.400	1:25	1:250	1:2500
.425	1:27	1:270	1:2700
.450	1:28	1:280	1:2800
.475	1:30	1:300	1:3000
.500	1:32	1:320	1:3200
.525	1:33	1:330	1:3300
.550	1:35	1:350	1:3500
.575	1:38	1:380	1:3800
.600	1:40	1:400	1:4000

Spleen extracts consisted of a so-called supernatant and cell residue. They were obtained as follows: 1.3 to 1.5 g of spleen was finely minced with scissors in 3 ml of saline in a small beaker set in ice. Saline to dilute the original tissue 1:10 was added in increments as the tissue was transferred to a centrifuge tube, and the supernatant, after adequate centrifugation, was poured off, inactivated and recentrifuged if necessary. The so-called cell residue was obtained by treating the minced spleen tissue residue in distilled water (1 part spleen as originally weighed plus 15 parts distilled water) in a micro-Waring blendor for 8 minutes. After standing at least half an hour in the refrigerator, the material was reconstituted to the proper isotonicity by adding 4 parts of concentrated saline. The mixture (final dilution 1:20) was inactivated and a supernatant obtained after centrifugation. The tissue was kept cold by using ice cold reagents, working over ice and wrapping the Waring blendor receptacle in carbon dioxide dry ice when in operation. Treatment of the spleen to obtain the supernatant did not decrease hemolysin but treatment to obtain the cell residue decreased it about 10% as determined by adding hemolysin of a known titer to normal spleen and carrying it through the above procedures. This loss was small as compared to the usual disparity between hemolysins in the spleen and serum.

Graphs in figure 1 through 6 were fitted by least squares when the points did not obviously fall on a straight line (cf. the decline in figure 5A). When one point was considerably out of line in a long series as, for example, on the 35th day in

figure 3C, it was disregarded in the calculations. Such aberrant points were remarkably few.

EXPERIMENTAL RESULTS

Hemolysins in normal rabbits.—Serums of rabbits were always titrated before immunization—usually just before injection. Normal serums in general gave a normal hemolysin titer of less than 1:50 and were often less than 1:1. A few exhibited titers as high as 1:125.

Titration of these serums before immunization, however, presented considerable difficulty. A series of tubes with increasing amounts of serum sometimes exhibited a zone phenomenon in which the percent of hemolysis would first increase and then decrease. When the values for a set of tubes crossed and recrossed the 50% value, the titer was considered to be that of the smallest amount of serum necessary and usually fell in a range of 1:1 to 1:50. Often, a series of dilutions exhibited a zone phenomenon but did not cross the 50% value even though large amounts of

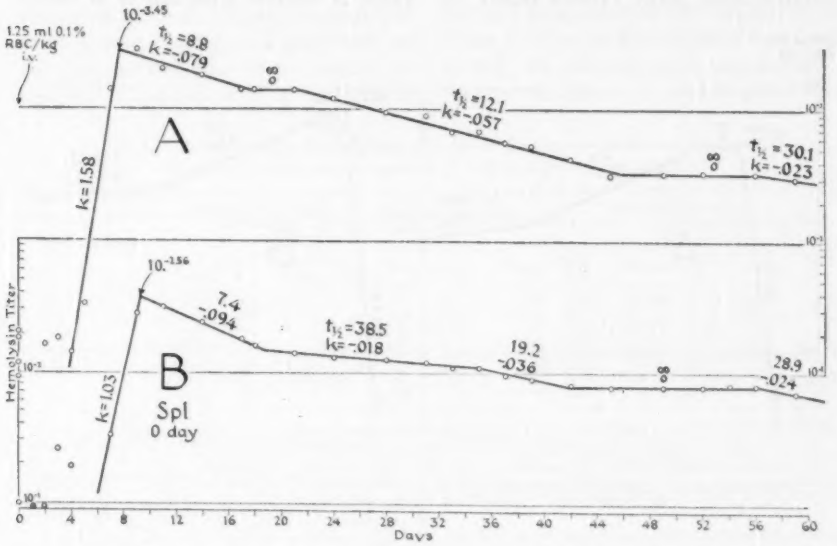

FIG. 1A.—Hemolysin titer in intact rabbit 73 which received 0.125 ml 1% sheep red cells intravenously.

FIG. 1B.—Hemolysin titer in rabbit 71 injected as was rabbit 73 and splenectomized on the same day.

Peak titers are given from the fitted curves. Hemolysin production during the initial rise is indicated by values of k (constant of increment), and successive rates of antibody decline are indicated by the half life in days. Titers shown on the 0 ordinate were obtained before immunization. Titers just after immunization were sometimes irregular and sometimes showed a negative phase as, for example, in figure 3A. They are not fitted to curves.

Hemolysin began to rise later in the splenectomized rabbit than in the intact rabbit, the rate of rise was less and the peak titer was lower. In both rabbits, the initial rise showed a constant rate of increase ($k = 1.58$ and 1.03, respectively). After the peak titer, the decline of hemolysin was rapid (half life of 8.8 or 7.4 days) for about 10 days in both rabbits with slower declines (half life of 12.1 or 38.5 days) of variable lengths of time (7 days to 25 days) interrupted by short plateaus (half life = ∞). For other data, see table 6.

undiluted serum were used. For these serums, the nearest value was considered to be the 50% unit, but the resulting titer—usually 1:1 to 1:20—was not considered very satisfactory.

Serums from several bleedings were titrated from many rabbits before injection, and 13 samples of blood were tested from three normal rabbits during the course of 1½ months. Hemolysin titers in these three animals varied from 1:22 to 1:50, 1:6 to 1:53 and 1:9 to 1:106, respectively.

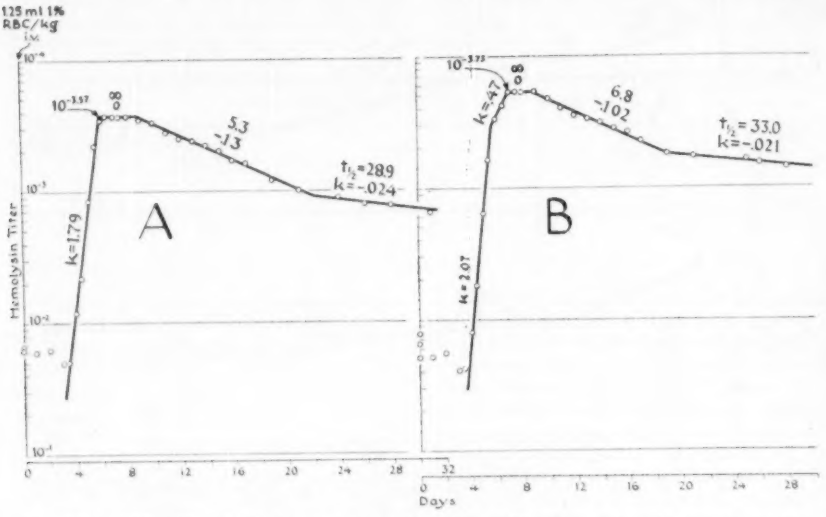

FIG. 2A and 2B.—Hemolysin titer in intact rabbits 91 and 92, respectively, which received 1.25 ml 1% sheep red cells intravenously. For other explanations, see figure 1.

Hemolysin began to rise on the 4th day. The rate of increase during the initial rise was high ($k=1.79$ and 2.07) in both rabbits. There was a second phase in both rabbits (a plateau for 2 days in 91 and $k=0.47$ in 92) and a third phase in 92 (a plateau of two days with $k=0$). The primary decline was rapid (half life of 5.3 and 6.8 days) for 14 or 11 days until a titer of 1:900 or 1:1800 was reached, and the secondary decline was definitely slower (half life of 28.9 or 33 days). For other data see table 7.

Antibody production in intact rabbits.—Following a single injection of sheep red cells into the marginal ear vein of rabbits, hemolysin titers of their serums fell on a series of essentially discontinuous curves described by the usual "compound interest" or monomolecular formula:

$$A_t = A_0 e^{kt}$$

where A_0 and A_t are antibody titers at time zero and t, respectively, and k is the constant of increment. Each decline in hemolysin titer is given as the apparent half life ($t_{1/2}=0.693/k$). This is a convenient method for comparing rates of decline although it is recognized (see Discussion) that it approaches the true half life of antibody only in some rabbits.

Three series of intact rabbits were studied following a single intravenous injection per kg rabbit of (1) 1.25 ml 0.1% standardized sheep red cells, (2) 1.25 ml 1% standardized sheep red cells, and (3) 1 ml 10% standardized sheep red cells, respectively. Standardization of the red cells is described in Materials and Methods.

Eight intact rabbits receiving 1.25 ml 0.1% cells/kg rabbit (table 6) gave peak hemolysin titers ranging from 1:2500

to 1:7500 on the 7th to 13th day except for atypical rabbit 4 described later. Although the antibody response in these 7 rabbits was highly variable as to peak titer, it was remarkably constant with regard to the antibody curve (fig. 1A). In general, there was no increase in the serum antibody before the third day. It then rose rapidly to a peak titer on the 7th or 8th day (fitted curve) in the first 4 rabbits with a constant of increment (k) varying from 1.04 to 1.58. In the other three rabbits there was a second phase of 2 to 5 days characterized by a value for k of 0 or 0.1. Once the peak was reached, the antibody began to decline, also uniformly, at rates which indicated a half life of the antibody globulin ranging from 3 to 8.8 days (mean of 5.5 ± 0.6 days). These rates continued for 6 to 22 days, usually from $1\frac{1}{2}$ to $2\frac{1}{2}$ weeks. A second slower antibody decline consisted of a short plateau for 3 to 5 days and a decline with a half life of 7 to 12 days (rabbits 75 and 73) or a period of consistent slow decrease with a half life of 18 to 42 days. These rates continued from 5 days to 32 days. A third antibody decline was more gradual than the second one in two rabbits (half life of 26.6 and 30.1 days) and more rapid in two others (half life of 6.2 and 16.2 days). The plateaus interrupting the antibody decline were so short that they may be considered as part of the immediately following decline. Thus, in figure 1A, the three declines interrupted by two plateaus may be considered as three declines with calculated half lives of 8.8, 14 and 43 days, respectively. Salient data on the 7 rabbits given this small dose of red cells may be found in table 6.

TABLE 6.*—*Hemolysin production, peak titer and decline for two months in intact rabbits following an intravenous injection of 1.25 ml 0.1 sheep red cells/kg.*

Rabbit no.	Figure	Maximum antibody production				Reciprocal of peak titer		Antibody decline					
		I		II				I		II		III	
		Days	k	Days	k	Day	$\times 10^3$	Days	Half life	Days	Half life	Days	Half life
75	1A	3–7	1.56			9	7.5	8–20	6.0	‡25–45	7.1	45–70	26.6
73		4–8	1.58			9	2.8†	8–18	8.8	‡21–46	12.1	‡56–76	30.1
2		3–8	1.3			10	2.5†	8–27	5.6	28–60	26.1		
1		3–7	1.04			10	2.8†	10–32	5.6	32–60	23.1		
74		3–7	1.61	7–9	0	7	7.0	9–15	5.0	15–21	31.5	22–43	6.2
9		3–7	.97	8–13	.1	13	6.3	13–22	3.0	22–32	18.0		
3		3–7	.94	7–11	0	10	5.6	11–22	5.7	23–28	42.0	29–60	16.2
4		3–8	.59			10	.4†	10–28	4.6	28–42	32.4	43–60	§

* Rate of maximum hemolysin production is indicated by the value of k (constant of increment) for the specified days after immunization. Antibody decline is indicated by the half life in days for the indicated days after immunization. All days refer to days after injection considering the day of injection as the 0 day. The rabbits are arranged in groups depending upon whether the period of maximum antibody production could be divided into 1-, 2- or 3-rate periods and within each of these groups in a decreasing order for k. A few rabbits were used for other experiments before two months and a few were observed more than two months. Data for maximal antibody production and antibody decline are based on fitted curves: data for peak titer on actual observations.

† Probably too low because of infrequent observations.

‡ This decline is preceded by a plateau of several days, i.e., the days intervening between this period and the preceding one.

§ A slight rise in titer with $k = 0.09$.

Rabbit 4, for which data are also given in table 6, was atypical in its behavior in that it showed a small value for k (0.59) during maximal antibody production with a low peak titer (1:400) and a secondary rise with a value for k of 0.09 from the 43rd through the 60th day. The peak titer, although probably too low due to insufficient readings, could not have exceeded 1:600 in view of the small value for k.

The sudden change at the peak in the first 4 rabbits in table 6 from a uniform rise to a uniform decline, which is characterized by a half life of the same order of magnitude obtained by other investigators for serum globulin, indicates that

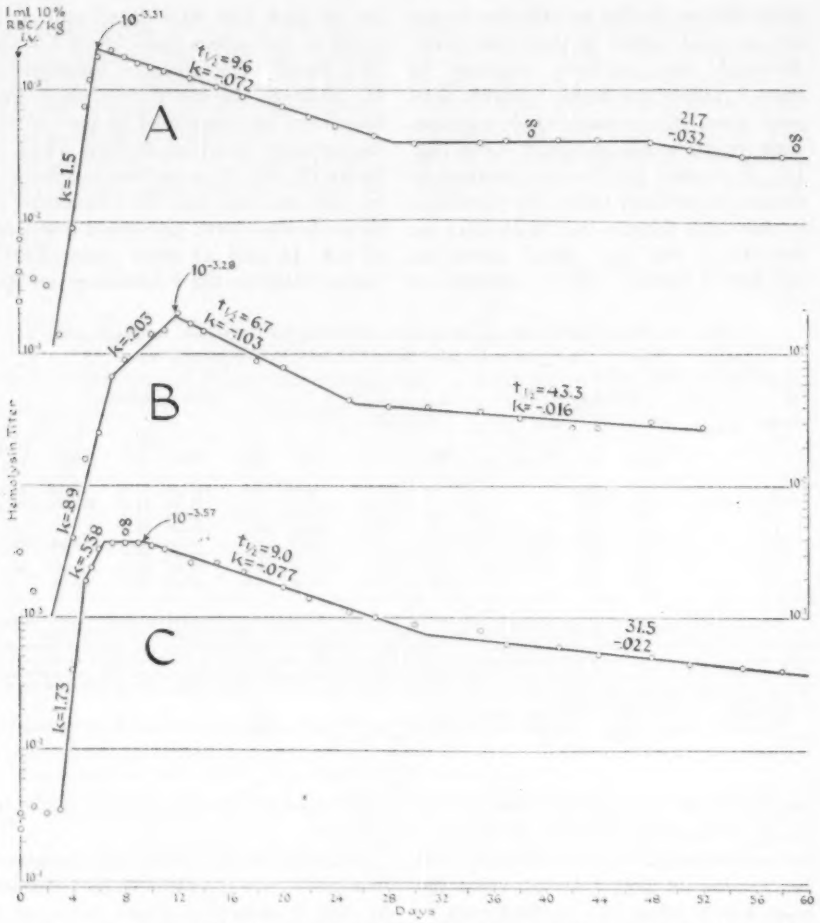

FIG. 3A, 3B and 3C.—Hemolysin titer in intact rabbits 106, 45 and 107, respectively, which received 1 ml 10% sheep red cells intravenously. For other explanations see figure 1.

Hemolysin had definitely risen in all rabbits on the 4th day. The initial rise was divided into one, two or three phases in the three rabbits. The decline was characterized by two or more rates of decline which gave successively larger half lives for the antibody. For other data see table 8.

antibody formation largely ceases. The fact that the peak was higher in these intact rabbits than in splenectomized ones and that the general decline often continued until the hemolysin titer reached the approximate magnitude observed in splenectomized animals (see later) indicates that the change is due largely to the cessation of splenic function.

Data from 17 rabbits receiving 1.25 ml 1% red cells, i.e., 10 times the antigen dose given to those in table 6, may be found in table 7. They are included primarily because they supplement the data just given on antibody half life

following the peak titer. The range in half life was essentially similar to the values obtained in the previous group (3.3 to 8.2 days). The average value was the same (5.5 ± 0.3 days). Some of the animals in this and the preceding group received two treatments of 400r hard X rays during the antibody decline (prel. rep., 1949, and to be reported later in detail), but the values shown in tables 6 and 7 were calculated on the antibody decline before X-irradiation.

The primary rise of antibody in this group was not usually followed immediately by a decline. In 11 rabbits, there was a secondary rise for about three days somewhat similar to that found in three of the rabbits in table 6 (cf. fig. 1A with fig. 2A). In rabbit 10 in table 7, there was a period of very slow decline from the 6th through the 17th day which is included in the category of secondary rise because antibody formation was continuing at a rate which almost, but not completely, equaled the rate of antibody destruction. The secondary rise characteristic of this group, which was associated with increased antigenic stimulation, indicates that the period of activity in the major antibody-producing system was active for three more days on the average. This period of longer activity was, however, at a much slower rate. Even more remarkable is the abrupt change in rate. The transitional phase must be less than half a day, i.e., the interval between consecutive readings (fig. 2A). In the last rabbit in this group, there was a 3rd change in rate. The value for k was 0 from the 7th to the 9th day after immunization (fig. 2B).

An antigen injection of 1 ml 10% red cells, i.e., 80 times that for the first group, was given to 29 rabbits. A summary of the results are given in table 8. Twenty of these rabbits were intact. Nine had various operations as controls for splenectomy (see next section). They are included here because their antibody response resembled that of the intact animals.

Although antibody titers in a few animals of this group rose to a peak and then declined in a manner already described for 4 rabbits in the first group

TABLE 7.*—*Hemolysin production, peak titer and decline for two months in intact rabbits following an intravenous injection of 1.25 ml 1% red cells/kg.*

Rabbit no.	Figure	Maximum antibody production				Reciprocal of peak titer		Antibody decline					
		I		II				I		II		III	
		Days	k	Days	k	Day	$\times 10^3$	Days	Half life	Days	Half life	Days	Half life
14		3–7	1.4			7	6.7	7–17	6.9				
11		4–7	1.4			10	2.1†	7–22	3.7				
17		3–7	1.26			7	1.5	7–16	5.3				
18		4–6	1.13			6	2.4	6–8	7.7				
19		3–6	1.12			7	.9	7–12	3.6				
5		3–6	2.76	6–13	.02	6	4.0	13–29	4.5	30–60	102.4		
91	2A	3–6	1.79	6–8	0	6	3.7	9–22	5.3	22–38	28.9	38–45	12.0‡
10		3–6	1.55	6–17	−.02§	6	5.2	17–22	4.2	23–60	16.9		
8		3–6	1.5	7–10	.03	7	3.1†	10–32	5.9	32–47	15.3	47–60	8.7
7		3–6	1.25	7–10	.01	10	1.8†	10–27	5.9	27–42	39.8	47–53	8.2
20		3–7	1.21	7–10	.08	10	8.0	10–17	6.0				
16		3–7	1.2	7–10	.03	10	4.0	10–17	8.2				
21		4–6	1.03	6–8	.06	8	.9	8–14	6.3				
6		3–7	1.01	7–14	.02	10	2.1†	14–32	5.2	32–60	39.4		
22		3–6	0.82	6–8	.02	9	1.2	9–12	3.3				
15		3–7	0.75	7–10	0	7	4.5	10–17	6.0				
92	2B	4–6	2.07	6–7	.47‡	7	5.3	9–19	6.8	20–38	33.0	38–60	22.3

* See first footnote in table 6.
† Probably too low because of infrequent observations.
‡ A plateau.
§ A slight decrease in titer with a half life of 29.1 days.

(fig. 3A and the first 5 rabbits in table 8), the majority showed a longer period of antibody formation similar to most of the animals in table 7. Thus, in 14 rabbits (6th through 19th in table 8), there was a clear-cut secondary rise at a much slower rate than the primary rise (fig. 3B). The value for k varied from 0.47 to 0.02. In rabbit 56, the primary rise was followed by a decline for 10 days which was so slow (half life of 17.3 days) that antibody was obviously being formed at a high rate. In addition, this slow decline changed into a more rapid decline with a half life (5.7 days) similar to the average value described earlier.

Finally, the period of active antibody formation could be divided into three stages in the last 9 rabbits in table 8 and is illustrated in figure 3C. In this figure, there was a primary rise for two days with $k = 1.73$, a secondary rise of one day with $k = 0.538$, a constant level of antibody for three days at a titer of 1:3700 and a decline for 21 days indicating a half life of 9 days. The third stage was characterized by a plateau lasting 3 or 4 days in three other rabbits in this group. The duration of any of these plateaus can be obtained from table 8 by subtracting the day terminating the second rise from the day beginning the primary decline. In the last 5 rabbits in table 8, the third stage exhibited slow declines for 4 to 9 days with the value for k varying from -0.02 to -0.05 and half lives of 14 to 42.5 days. These slow declines were then followed by the characteristic rapid decline giving a half life of 5 to 6.7 days.

The average half life of the primary decline was 7.95 ± 0.41 days for the 20

TABLE 8.*—*Hemolysin production, peak titer and decline for two months in intact and operated control† rabbits following an intravenous injection of 1 ml 10% red cells/kg.*

Rabbit no.	Figure	Maximum antibody production				Reciprocal of peak titer		Antibody decline					
		I Primary rise		II and III Secondary rise				I		II		III	
		Days	k	Days	k	Day	$\times 10^6$	Days	Half life	Days	Half life	Days	Half life
29		3–5	2.1			5	4.0	5–18	8.1				
106	3A	3–6	1.5			7	2.0	6–28	9.6	‡48–54	21.7	‡	
108		3–6	1.4			6	2.0	6–35	10.1	35–62	26.6		
30		3–5	1.37			7	.4	6–18	7.7				
49		2–5	1.35			7	1.6	5–32	8.0	33–46	130.0		
111		3–5	3.11	5.7	.20	7	6.0	7–11	5.0				
81†		4–6	2.64	6–8	.06	8	5.3	9–33	7.1	33–60	18.7		
79†		4–6	2.33	6–9	.07	9	6.2	9–29	6.6	29–61	10.2		
103		3–5	2.23	5–8	.16	8	3.0	9–30	8.0	30–39	27.8		
87†		4–5	2.06	6–8	.14	8	2.1	9–25	7.7	‡32–42	11.5	43–63	27.8
110		3–5	2.04	5–7	.02	7	3.6	7–17	6.9	17–33	10.5	‡41–62	18.2
57		3–5	1.83	5–9	.09	9	3.1	9–31	6.4	31–48	44.4		
109		3–5	1.8	6–7	.47	7	6.0	7–29	7.3	29–53	18.7	53–62	69.3
60		3–5	1.25	5–9	.20	9	1.2	9–21	11.9	21–99	50		
43		3–5	1.16	5–10	.14	10	1.6	10–31	8.8	32–52	∞		
28		3–6	1.15	6–9	.20	9	2.2	9–12	4.7	‡21–23	13.6		
58		3–6	1.01	7–10	.21	11	2.1	10–17	10.3	17–50	13.8	‡67–75	16.9
45	3B	3–7	.89	7–12	.20	12	2.0	12–24	6.7	25–52	43.3		
59		2–5	.67	5–9	.16	9	.6	9–14	10.0	14–60	43.3	‡	
56		3–6	1.19	6–16	−.04	7	3.0§	16–27	5.7	‡34–48	8.5		
85†		4–5	2.03	6–9	.15‡§	9	2.2	13–27	7.5	27–42	17.3	42–63	70.4
107	3C	3–5	1.73	5–6	.54‡§	7	3.7	9–30	9.0	30–62	31.5		
88†	4C	3–5	1.65	6–9	.38‡§	13	5.6	13–26	5.9	27–63	18.7		
112		3–5	1.53	6–8	.06‡§	8	6.0	11–27	8.0	27–44	49.5	44–62	14.4
90†		3–4	3.08	5–8	.27§	9	10.0	15–35	6.7	35–63	45.3		
89†		3–4	2.83	5–9	.13§	9	8.4	13–25	6.0	25–40	10.7	40–63	29.9
86†	4B	3–5	2.55	5–9	.39§	9	10.6	15–27	6.0	27–56	14.4	56–63	33.0
113		3–5	1.96	6–7	.11§	7	2.6	16–27	6.7	‡39–62	21.0		
82†	4A	4–6	2.35	6–9	.26§	9	8.9	17–26	5.0	26–55	10.8	55–64	25.7

* See first footnote in table 6.
† As a control for the splenectomized rabbits, 9 of these rabbits had various organs removed on the day of injection (see text).
‡ Plateau for several days or more.
§ The last 9 rabbits in this table had a third change in rate as follows: In the first 4 rabbits, there was a plateau or practically a plateau for 3 or 4 days and in the next 4 rabbits, there was a half life of 14, 42.5, 15, and 16 days for 6, 4, 6, and 9 days, respectively. For rabbit 82, see figure 4A.

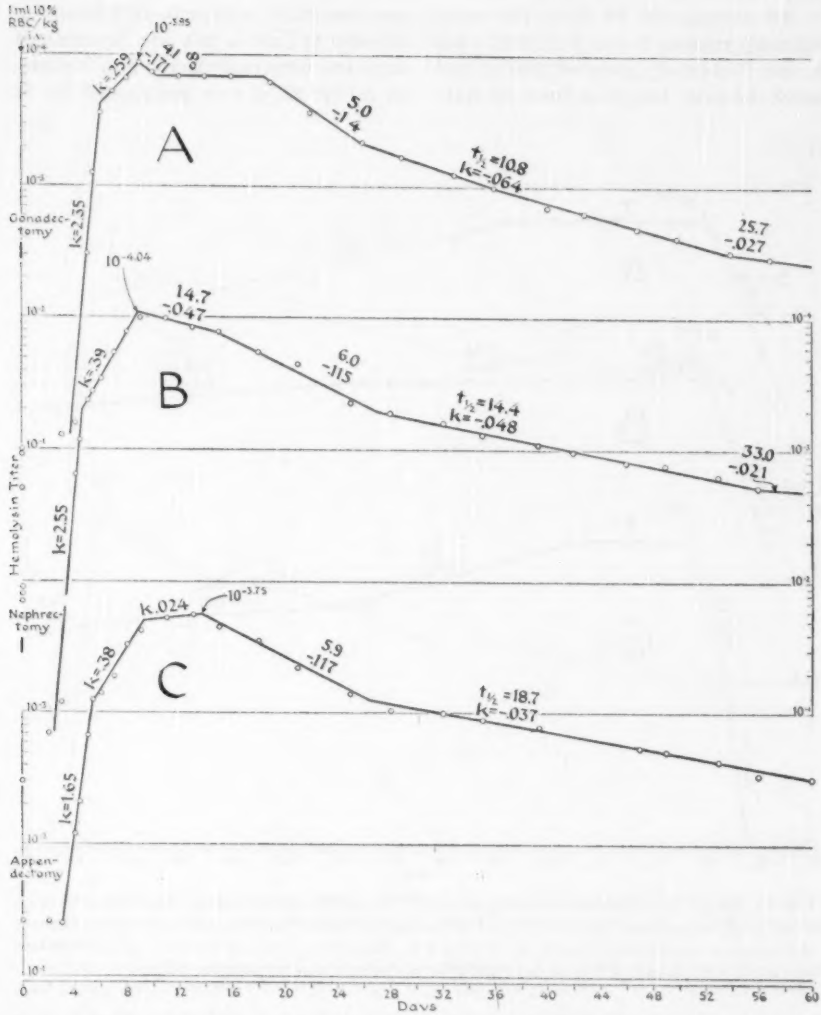

FIG. 4A, 4B and 4C.—Hemolysin titer in rabbits 82, 86 and 88 from which one testis, one kidney and the appendix were removed, respectively, on the day of an intravenous injection of 1 ml 10% sheep red cells. For other explanations see figure 1.

After the peak titer in 4A, there was a rapid decline (half life of 4.1 and 5 days) for 2 and 10 days interrupted by a plateau of 6 days. After the peak titer in 4B there was a slow decline (half life of 14.7 days) for 6 days followed by a rapid decline (half life of 6 days) for 14 days. Otherwise the rise and fall of the hemolysin titer in these rabbits were essentially similar to one or the other of the graphs shown in figure 3. For other data see table 8.

intact rabbits in table 8. It continued on the average for 16 days. For some unknown reason, it was 6.5 ± 0.32 days in the 9 control operated group and lasted the same length of time. In spite

On the other hand, the second decline was essentially similar in all 3 groups of rabbits. It gave a half life of about 31 days and frequently lasted 3 to 5 weeks. In rabbit 60, it was maintained for 11

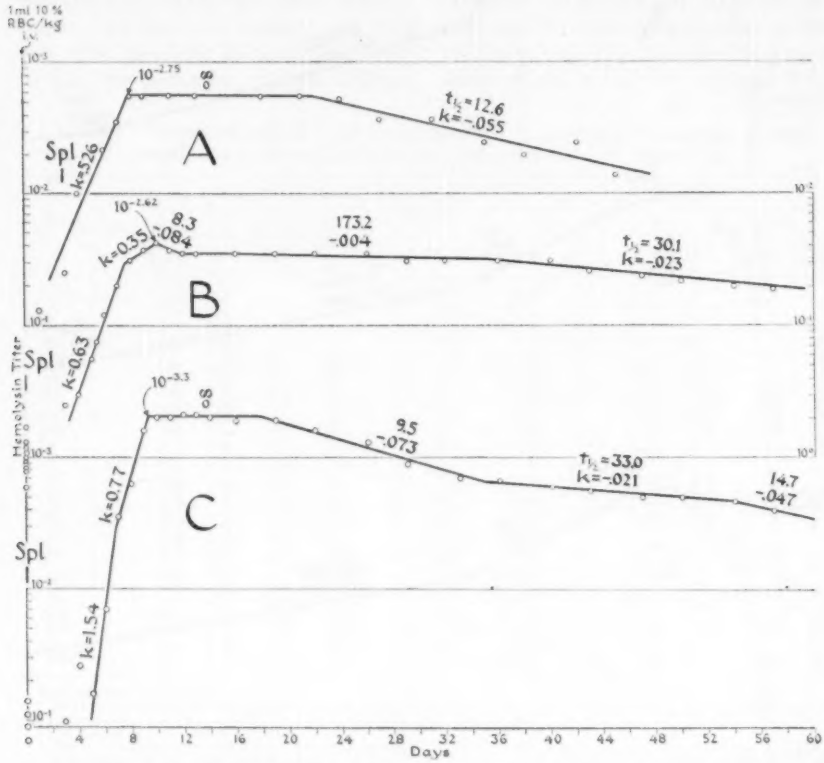

FIG. 5A, 5B and 5C.—Hemolysin titer in rabbits 47, 83 and 80 splenectomized three days after or on the day of an intravenous injection of 1 ml 10% sheep red cells. For other explanations see figure 1. Hemolysin accumulated gradually ($k = 0.53$ to 1.5). Peak titers in two of the three rabbits was low. There was a long plateau of 9 to 24 days just after the peak. For other data see table 9.

of this discrepancy, the half life of antibody decay during the primary decline was longer and the decline lasted about three days longer in the rabbits of table 8 than in those of tables 6 and 7. The longer period of over-all production and primary decline of antibody indicate that antibody was being produced at a higher rate for a longer period.

weeks. The half life of the third decline was often even longer than that of the second decline and was still being maintained in some rabbits at the end of the period of observation. Plateaus were sporadically associated with these declines viz., in rabbits 106, 87, 28 and 56 before the second decline and in rabbits 29, 110, 58 and 59 before the third

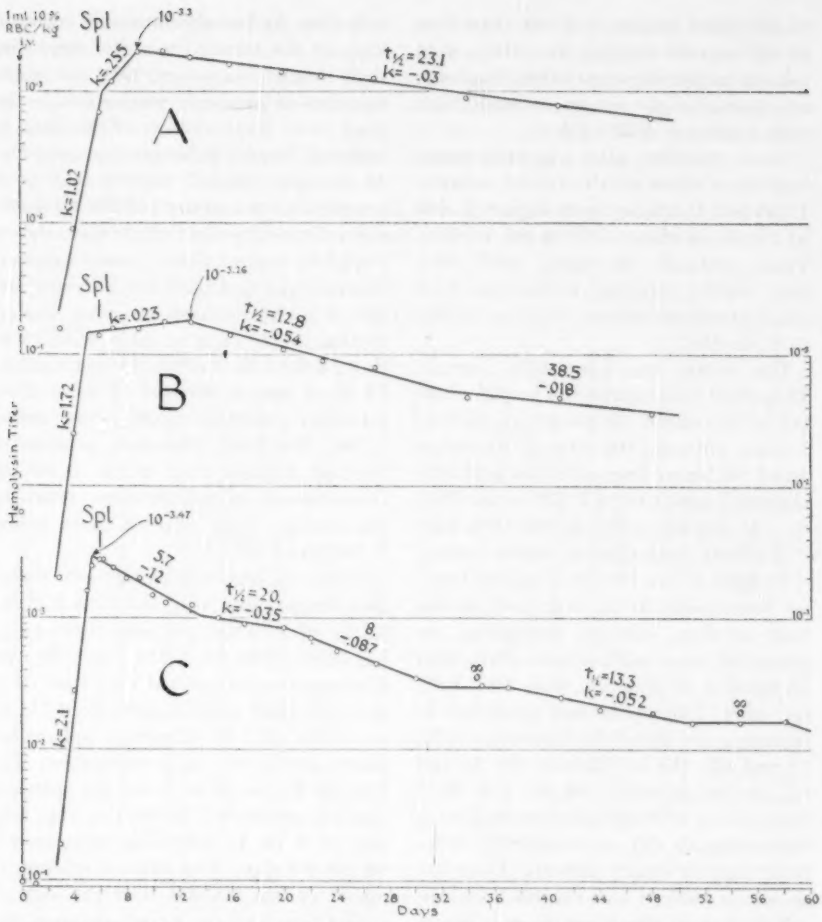

FIG. 6A, 6B and 6C.—Hemolysin titer in rabbits 52, 51 and 100, respectively, which were splenectomized 6 days after an intravenous injection of 1 ml 10% sheep red cells. For other explanations see figure 1.

Hemolysin began to decline in 6C immediately after splenectomy (half life of 5.7 days). It increased after splenectomy in 6A and 6B and then began to decline very gradually. For other data see table 9.

decline. They lasted from one to three weeks.

Briefly, hemolysin titers for 7 weeks after the peak in all nonsplenectomized rabbits generally followed a series of discontinuous semilogarithmic curves with decreasing values for *k* (fig. 3B and 3C). Thus, in 21 of 30 rabbits (tables 6 through 8) which were studied for 6 weeks or more, the rate of decline was broken into two or three successively more gradual declines. In the remaining 8 rabbits, the declines in hemolysin titer were interrupted by plateaus or slight declines of several days to three weeks. Occasionally, the rate

of the third decline was less than that of the second decline. In rabbit 4 in table 6, as already mentioned, there was an unusual slight rise in antibody titer with a value of 0.09 for *k*.

Two months after immunization, hemolysin titers usually varied between 1:100 and 1:350, but were higher (1:400 to 1:600) in about 20% of the rabbits. They gradually decreased until they had usually returned within the high range of normal rabbits (1:50 to 1:100) in 5 months.

One rabbit was essentially anergic. In spite of being given the largest number of red cells at the same time as were 5 other animals, the titer of its serum was 1:90 before immunization and only reached a peak titer of 1:120 on the 10th day. It was discarded on the 18th day.

Antibody production in control rabbits.—In view of the effects of splenectomy on hemolysins to be described in the next section, control operations on groups of three rabbits were performed on the day of injection with 1 ml 10% red cells. The operations consisted in removing one testis via laparotomy (79, 81 and 82), the left kidney (85, 86 and 87) or the appendix (88, 89, and 90).* The course of hemolysin production in these animals did not essentially differ from that in intact animals. Data are shown in table 8 and results from one rabbit in each group are given in figure 4. Figure 4B illustrates a slow decline from the 9th to the 15th day characteristic of the third phase of antibody production in the last 5 rabbits in table 8.

Antibody production in splenectomized animals.—Antibody production in splenectomized animals was not only generally less in amount but was generally at a slower rate if splenectomy was performed near the time of antigen injection. As hemolysin rapidly accumulates in the serum for a few days and then slowly disappears, the best single measure of antibody production is the peak titer. Examination of the data in tables 6, 7 and 8 indicates that only 5 of 54 nonsplectomized rabbits had peak hemolysin titers under 1:1000. Of these, one and possibly more might have shown a slightly higher titer if more frequent observations had been made during the period of high antibody response. Examination of the data in table 9 indicates that most of the rabbits splenectomized 14 days before through 4 days after injection exhibited peak titers under 1:700. We have, therefore, arbitrarily selected a peak titer below 1:700 as characteristic of splenectomy. Actually the average peak titer of those below 1:700 was 1:390.

Three rabbits splenectomized about two hours before injection with 1.25 ml 0.1% red cells had low peak titers ranging from 1:90 to 1:310 (fig. 1B). In the longer series injected with 1 ml 10% red cells, peak titers ranged from 1:120 to 1:560 in 3 of 5 rabbits splenectomized on the day of immunization (fig. 5B and C), in all of 6 rabbits splenectomized on the 3rd or 4th day (fig. 5A) and in 2 of 4 rabbits splenectomized on the 5th day. Two rabbits splenectomized on the 5th day with the highest peak titers may also have been partially affected by splenectomy, since hemolysins had already reached a titer of 1:500 or 1:600 at the time of splenectomy and their peak titers of 1:1200 and 1:1500 were lower than all but one rabbit splenectomized on the 6th or 8th day. Splenectomy was effective in 2 rabbits when performed 2 weeks before injecting the antigen, in 1 of 3 rabbits when performed 4 weeks before, and in none of 3 rabbits when performed 14 weeks before. When carried out 6 and 8 days after the injection of antigen, it was

* We are indebted to Prof. Lester Dragstedt for performing the appendectomies.

without demonstrable effect on antibody production. This was to be expected as the titer was already high when splenectomy was performed except in rabbit 105.

One possibly anergic rabbit (38) was the basis for the ensuing description. Although the values for k in rabbits showing marked effects of splenectomy overlapped the value for this constant in intact animals, its mean value was lower. It ranged from 0.04 to 1.67 with

TABLE 9.*—*Hemolysin production, peak titer and decline for two months in splenectomized rabbits. The rabbits were splenectomized 98 days before to 8 days after an intravenous injection of sheep red cells*†.

Rabbit no.	Figure	Day when splenectomized	Maximum antibody production				Reciprocal of peak titer		Antibody decline					
			I		II				I		II		III	
			Days	k	Days	k	Day	× 10^3	Days	Half life	Days	Half life	Days	Half life
78		98	3–7	1.5			9	6.3	9–14	5.2	14–33	10.7‡	42–49	12.6‡
77		98	3–7	1.81	7–9	.64	9	6.3	9–15	5.3	15–45	13.9	45–60	99.0
76		98	3–7	1.92	7–8	.65‡	9	7.9	10–15	4.1	15–43	15.1	43–60	28.9
97		28	5–8	1.05	9–11	.02	9	1.1	11–42	13.8	42–50	77.0		
96		28	5–8	1.03	8–11	.05§	11	1.4	25–37	11.7				
95		28	5–8	.61	8–10	.15	10	.35	10–14	10.0‡	23–	49.5		
99		14	6–8	.8	9–10	.10	10	.31	10–16	8.9				
98		14	4–6	1.91	7–9	.09‡	9	.62	22–49	27.7	49–62	17.3		
72†		0	5–7	1.67			8	.3	7–17	5.2§	33–44	14.4‡	58–66	6.7
71†	1B	0	6–9	1.03			11	.31	9–21	7.4‡	35–42	19.2‡	56–66	28.9
70†		0	5–9	.52			9	.09	9–21	10.0‡	45–52	26.7‡		
42		0	6–8	.65			10	.37	10–14	10.9‡	23–38	21.7	38–52	15.1
84		0	5–7	1.76	7–9	.55	10	2.8	10–13	8.2‡	19–26	7.2	26–54	28.9‡
83	5B	0	4–7	.63	8–10	.35	10	.42	10–12	8.3‡	36–64	30.1		
80	5C	0	5–7	1.54	7–9	.77‡	10	2.0	18–35	9.5	33–54	33.0	54–64	14.7
46		0	7–12	.26	13–38	0	14	.24	40–52	5.6				
34		3	6–12	.16			12	.12	12–16	6.8	16–22	k = .09§		
39		3	4–8	.45	8–28	0	8	.31	28–42	19.0‡	48–52	15.2		
47	5A	3	3–8	.52	8–20	0	8	.56	21–45	12.6				
38		4	4–11	.04			11	.04	11–22	10.8	22–52	k = .03§		
32		4	3–5	1.4	5–8	.04	8	.21	9–19	5.8				
31		4	4–7	.54	7–21	.03	21	.53						
40		5	4–7	.43			8	.3	7–12	6.9	12–52	51.4		
37		5	3–5	1.46	5–9	.48	7	1.2						
41		5	3–6	1.0	7–25	− .03	7	1.5	25–33	9.0	34–48	33.8		
36		5	3–5	1.32	5–8	.34‡	10	.5	16–22	18.4				
54		8	3–4	1.80	5–7	.46	7	3.5	7–16	4.4	16–48	35–7		
100	6C	6	3–5	2.1			6	2.8	6–14	5.7	13–20	20.0	20–31	8.6
104		8	3–5	3.3			7	4.0	6–29	6.8	29–62	18.7		
105		8	4–5	.98	6–8	.14	8	.45	8–24	8.6	24–44	16.3	‡55–62	11.0
53		8	3–5	2.2	5–8	.19	8	6.3	8–36	9.7	36–48	14.3		
101		6	4–6	1.93	7–9	.08	8	2.0	9–16	5.8	16–29	11.9	29–62	28.8
52	6A	6	3–6	1.72	6–13	.02	11	1.8	13–33	12.8	33–48	38.5		
50		6	4–6	1.1	7–25	− .04	9	2.0	25–37	8.7	30–48	‡		
55		8	4–6	1.15	7–18	.05	16	2.8	18–48	10.1				
51	6B	6	3–6	1.02	7–9	.25	9	2.0	9–48	23.1				

* See first footnote in table 6. Rabbits 80, 83 and 84 were splenectomized 3 hours after immunization.
† Rabbits 70 to 72 were injected with 1.25 ml 0.1%; all others with 1 ml 10%.
‡ Plateau for several days or more or a very slow decline.
§ A slight rise in titer.

encountered among the splenectomized series. It gave a preimmunization titer of 1:33 and never showed a titer of >1:45 for 52 days. It was injected with 1 ml 10% cells and was splenectomized on the 4th day after immunization. Its hemolysin titer gradually increased to 1:42 on the 11th day, declined to 1:22 by the 25th day, and increased again to 1:45 by the 45th day.

Data from all splenectomized rabbits are summarized in table 9 and form an average of 0.64 in 12 rabbits which were markedly affected by splenectomy performed from 0 through 4 days. In addition, a primary rapid decline was only typical for the splenectomized rabbits (rabbits 70, 71 and 72) receiving the small amount of antigen (fig. 1B). Rabbits splenectomized from 0 through 4 days after receiving 1ml 10% cells (except rabbits 80 and 84) not only showed a slower rise but generally a slower decline from the peak than did intact

animals. Even when the decline was similar in rate to that in the normals, it lasted a shorter time and then changed to a slow decline (fig. 5B). In others, a plateau occurred after the peak and lasted for several weeks (rabbits 46, 39 and 47). The slow decline was on the average at the same rate ($t_{1/2}=26$ days) and lasted for about the same period (29 to 47 days) as the secondary decline in intact animals (31 days for 29 is not significantly different (1.1 ± 2.6) from the rabbits splenectomized earlier (table 10).

In animals splenectomized from 0 through 4 days after immunization, there was a more sustained antibody production in animals receiving the large amount of antigen (1 cc of 10% cells) than in those receiving the small amount (0.125 cc of 1% cells). Thus, the antibody curve in rabbits 70–72 after this

TABLE 10.—*Half life of hemolysin in days during the primary decline after the peak titer in nonsplenectomized rabbits and in rabbits splenectomized 14 days before to 8 days after one intravenous injection of sheep red cells (data from tables 6 through 9).*

Half life of hemolysin in days	Antigen dose					
	0.125 and 1.25 ml 1%	1 ml 10%		0.125 ml 1%	1 ml 10%	
	Intact		Operated controls	Splenectomized		
				14 days before through 5 days after		6 and 8 days after
3	2					
4	4		1			1
5	7	2		1		2
6	7	3	3		2	2
7	2	3	4	1	2	1
8	2	5	1		2	
9	1	2			2	1
10		4		1	1	1
11					2	
12		1				1
>12					5*	1†
Mean	5.5 ± 0.3	7.95 ± 0.4	6.5 ± 0.3	7.5 ± 1.6	10.6 ± 2.0	9.5 ± 1.7

* In these five rabbits, the half life was 13, 18, 19, 21 and 28 days.
† In this one rabbit, the half life was 23 days.

to 53 days). The declines were sometimes interrupted by plateaus. Plateaus in these and in nonsplenectomized rabbits may indicate that successive nonsplenic sources cease to produce antibody periodically.

In the first 6 of the 10 rabbits splenectomized 6 to 8 days after immunization, the titer began to decline immediately after the operation (fig. 6C) whether the primary rise showed a constant rate of increase or two discontinuous ones (table 9). In 3 of the last 4 rabbits in this group, the titer after splenectomy rose either rapidly (fig. 6A) or slowly (fig. 6B) and then declined slowly, especially in the last rabbit in table 9 with a half life of 23.1 days (fig. 6A). The half life of the primary decline in this group, however, small amount consisted of a single rise and an immediate decline which lasted from 10 to 12 days, with an indicated half life of 5.2 to 10 days (fig. 1B and table 9). The antibody curve in the 11 rabbits after the large amount was similar in only 34, 38 and 42: the others had a secondary rise or a plateau after the peak (fig. 5 and table 9). Similar evidence is found in the generally slower decline in animals receiving the larger amount of antigen.

The abnormally long continued slow rise of antibody and its maintenance at a level titer in some splenectomized animals are used in the schematic representation of the relation of serum antibody arising from the spleen and from nonsplenic sources in figure 7. The figure does not represent average results

but is a generalization derived from the following considerations. The average peak titer of all intact animals given 1 ml 10% cells was 1:3980 and of all animals splenectomized on 0 through 4 days after receiving the same antigen dose was 18% or 1:718. Omitting two rabbits (80 and 84) with unusually high titers, the splenectomized average was 1:354.

titrations were made to ascertain the amount of hemolysin (1) in the serum, (2) in washings from the spleen when coarsely minced in saline (supernatant), and (3) in the spleen residue when further minced by the Waring blendor in distilled water (cell residue). Preparation of the spleen is explained in Materials and Methods.

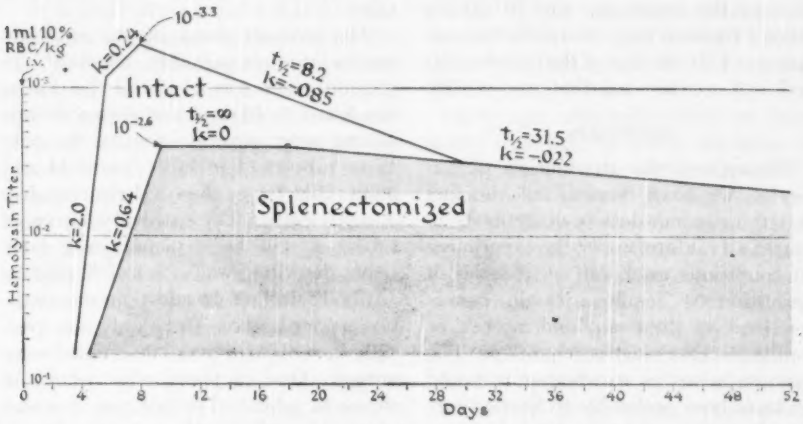

FIG. 7.—Schematic representation of total hemolysin titer separated into that produced by the spleen (clear area) and by other sources (stippled area) as shown by results from intact and splenectomized rabbits after the injection of 1 ml 10% sheep red cells. Data were obtained from intact rabbits showing 2 phases of the initial rise and from rabbits splenectomized on the day of immunization showing a long plateau or slow decline after the peak titer. For other explanations see figure 1.

Hemolysin from nonsplenic sources was about 20% of the total peak titer but continued to be produced for long periods, whereas that from the spleen was restricted to the first week of immunization. The two increased and decreased in the serum at different rates for different periods.

We have, therefore, used a peak titer of 1:400 for splenectomized animals and a peak titer of 1:2000 for the intact animals. This procedure utilized the 1:5 ratio of the over-all averages with the approximate peak serum antibody value for animals showing the usual effect of splenectomy. The values of *k* are approximations from splenectomized animals and from typical intact animals showing the common two-phase rise.

Splenic content of antibody.—The preceding results establish the importance of the spleen in hemolysin production. Hemolysin titer of the spleen was, therefore, studied. For this work, parallel

The titrations involved 56 rabbits injected for 1 to 18 days with 1 ml 10% red cells. Detailed results will be presented in a later paper (prel. rep., 1950a and b). In general, hemolysin titer was higher in the residue than in the supernatant but not as high as in the serum, except sometimes on the 3rd and 4th day of immunization when titers were low (rarely above 1:100) and somewhat unreliable. Furthermore, the spleen-weight:body-weight ratio of these rabbits was not definitely higher at 3 and 4 days after immunization than at other times. It usually varied between 0.04 and 0.065% body weight. The rise of

hemolysins in the serum was reflected by a rise in the spleen, but was not only less but relatively more variable in the spleen. Thus, titers in the spleens of the cell extract and supernatant residues together varied from <1% to 50% of the serum on the 6th and 8th days in 10 rabbits. They were around 20% on the average thereafter. The mean hemolysin titer of the serum for the 56 rabbits killed 1 through 18 days after immunization was 1:1010; that of the supernatant and cell extract together was 1:280.

DISCUSSION

Throughout the description of our results, we have emphasized the fact that the rise and decline of antibody in almost all rabbits, under the experimental conditions used, fall on a series of discontinuous semilogarithmic curves described by the compound interest or monomolecular reaction equations. The question arises as to whether it would not have been preferable to fit the titers of antibody rises, such as shown in figure 3C, to a curved line rather than the series of straight lines shown. Plots of antibody rise may have been sigmoid in a few rabbits, but almost invariably fitted straight lines better. The discontinuity of the different phases of the antibody rise is particularly well shown in the closely spaced determinations in figure 2. Somewhat similarly, plots of antibody decline practically always fell on a series of straight lines as shown in the figures. A minor point with respect to fitting the curves is the short length of some of the plateaus. Thus, in figure 1A, data points from the 8th to the 46th day could have been fitted on a single line without the plateau. The value of k would then be intermediate. The abruptness of the changes in velocity (k) must be interpreted in light of the fact that the closest observations were a half day apart when k was expected to change during the initial rise. During this time, a few readings fell in a transitional position on the curve, as is seen in figure 2B at the beginning and end of the plateau at the peak titer. The rest fell on one or the other of the intersecting lines, as is best illustrated in figure 2A by the four readings as the acute rise changed into the plateau and the plateau changed into the primary decline.

The over-all shape of the antibody curves was remarkably constant. In general, there was an initial rise during the first 7 to 10 days and then a decline lasting over several months. In only three rabbits (4 in table 6 and 34 and 38 in table 9) was there a slight rise after 3 to 7 weeks. The antibody curve of rabbit 4 will be depicted in a later paper describing the effect of X rays on antibody decline. In addition, most rabbits were reactive. Thus, only one, possibly 2, rabbits of over 100 studied were anergic. One of these was rabbit 38 shown in table 9. The late rise in it was of questionable significance since it was so small and the titer so low.

A comparison of the peak titers in animals splenectomized on the day of immunization through the 4th day (when serum antibody begins to rise precipitously) leaves little doubt that the spleen is the major antibody-forming unit during the initial antibody rise in most cases (12 of 14 rabbits in table 9). The exceptions are important and will be dealt with later. The data also indicate that the initial decline begins in intact animals when a major antibody unit, most probably the spleen, ceases abruptly to form antibody. The latter conclusion is indicated by (1) the decline is described by the equation for a monomolecular reaction, (2) splenectomy about the time of peak titer does not cause a more rapid decline—which should occur if the spleen were continuing to function at a high level, and (3) the decline indicates a half life in the

order of magnitude accepted for serum globulin. These points are discussed in detail in the following paragraphs. Before beginning this discussion, it should be emphasized that, although the spleen ceases to form antibody at the beginning of the rapid decline, nonsplenic sources continue to produce antibody over long periods.

It is generally assumed that a serum protein undergoing metabolic degradation without continuing synthesis should disappear at a constant logarithmic rate. These conditions should be ideally satisfied when homologous proteins are passively transferred to animals. Nevertheless, recent experiments with antibody and isotopically labelled proteins have not given uniform logarithmic decreases. Thus, neither Heidelberger et al (1942), studying the passive transfer of rabbit antipneumococcus antibody in the rabbit, nor Fink et al (1944), using isotopically labelled homologous plasma proteins in the dog, found a uniform rate of disappearance but a rapid initial removal with a progressively slower disappearance. Fink et al suggest that the early relatively rapid loss of passive antibody from the circulation may be due to the escape of antibody into the tissues with a return of an equivalent amount of protein to the blood.

In marked contrast to these recent studies are the findings of Mason et al (1930) on the decline of lamb dysentery antitoxin passively transferred from the ewe to the lamb in colostrum. This passive antibody in the lamb disappeared at a uniform logarithmic rate whereas antibody in the actively immunized ewe disappeared at a progressively slower rate (see also review in Burnet and Fenner, 1949).

Data for the first 6 rabbits splenectomized 6 and 8 days after immunization in table 9 and figure 6C indicate that the half life of antibody during the initial decline is within the range of intact animals. Hence, the spleen does not form much antibody after the peak titer is reached. Data for the last 4 rabbits in table 9 and figures 6A and 6B indicate that hemolysin may increase after splenectomy and decay at a slower rate. Hence, nonsplenic sources may form antibody in appreciable amounts. This conclusion is borne out by the cessation of titer in 2 of 8 rabbits splenectomized on the day of immunization (fig. 5C).

Although determinations of antibody half life by other workers are scarce, our data are within the order of magnitude reported. As described previously, the half life value for the primary antibody decline was the same for the rabbits receiving the two smaller doses of antigen. Thus, of the rabbits receiving 1.25 ml 0.1% and 1.25 ml 1% cells, it was 5.54 ± 0.57 and 5.52 ± 0.26, respectively. The data from these two groups are combined in table 10. The animals receiving 1 ml 10% red cells gave a longer half life ($t_{1/2} = 7.95 \pm 0.41$) for the primary decline. The difference between these two groups is 2.43 ± 0.48 days for which $P < 0.001$.

The significantly longer half life during the primary decline following the injection of the largest amount of antigen is probably due to a high continuing activity of nonsplenic sources rather than to a continuing function of the spleen as indicated by the more sustained antibody formation in splenectomized animals receiving the large amount of antigen (see page 52). It can also be visualized in intact animals by a hypothetical case.

Assume, for example, an initial peak antibody titer of 1:2000 for both the small and large amounts of antigen, that 80% of this is from the spleen and 20% from other sources, that antibody sources other than the spleen maintain

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Assume, for example, an initial peak antibody titer of 1:2000 for both the small and large amounts of antigen, that 80% of this is from the spleen and 20% from other sources, that antibody sources other than the spleen maintain

their 20% level ($t_{1/2} = \infty$) only after the increased antigenic stimulation of the large amount of red cells, and that the half life of over-all serum antibody is 6 days ($k = -.115$) after the small amount and 8 days ($k = -.087$) after the large amount. From these assumptions, it would follow: With the small antigen dose, the decline of splenic and nonsplenic antibody would be identical ($k = -.115$ and $t_{1/2} = 6$ days) [redacted] large dose, the original titer of 1:2000 would have decreased to 1:1000 in 8 days (by assumption) but, of this, 1:400 would be nonsplenic ($k = 0$ and $t_{1/2} = \infty$), whereas 1:600 would be splenic antibody or only 60% of the total antibody instead of 80% as at the peak. The value for k of splenic antibody alone during this period would, therefore, be $-.125$ with $t_{1/2} = 5.65$ days. Consequently, although the assumed over-all half life was 25% greater with the large dose than with the small dose, the half life of splenic antibody would be about the same.

In the previous computations it must be kept in mind that a regular logarithmic decrease of total antibody with a constant level of antibody from nonsplenic sources would give a curved line for splenic antibody alone. The line would be approximately straight at first, however, and would curve more rapidly as splenic antibody reached zero. In the hypothetical example given above, the plot for splenic antibody would be essentially linear for the first 8 days. Another interesting fact is that a comparatively minor decrease in nonsplenic titer after about the 8th day would straighten out the over-all serum antibody curve on a semilogarithmic plot. This situation largely holds in the schematic curves in figure 7. In this figure splenic antibody (antibody in intact minus that in splenectomized rabbits) approximates a linear function from the 8th to the 22nd day because of the decline of nonsplenic antibody in the splenectomized rabbits beginning on the 17th day. Such considerations indicate that the half life values following the injection of the smaller amounts of antigen in table 10 are the most convincing.

Our conclusion that increases in the antigenic stimulation tend to increase the activity of nonsplenic antibody sources is in line with the hypothesis advanced by Motohashi (1922) to account for his finding that the effectiveness of splenectomy in reducing hemolysin production was apparent with small but not with large doses of antigen. He suggested that with small doses a large proportion of the antigen is taken up by the spleen which then forms most of the antibody whereas with large doses of antigen so much is taken up by the liver and bone marrow (and possibly other tissues) that the spleen ceases to be a determining quantitative factor.

Schoenheimer et al (1942) found that the concentration of isotopically labelled nitrogen in antibody in the rabbit decreased to half its maximum ratio in about two weeks. This length of time, therefore, they considered to approximate the half life of the antibody molecule. It would be outside the upper limit of our values. Their figures, however, when plotted semilogarithmically seem to indicate a half life of 10 days. This interval would fall within our range. More recently, Libby and Madison (1947, see also Salley and Libby, 1947), in studying antibody response to isotopically labelled tobacco mosaic virus in mice, found that circulating antibody decreased gradually from the 8th to the 31st day after immunization and concluded that it approximates a linear relationship from the 12th day when plotted logarithmically against time. Although their data points, which were based on pooled serums from five mice, show a wide scatter, they calculated antibody half life as approximately one week and explained the

smaller value as compared to the results by Schoenheimer et al on the higher rate of metabolism in the mouse as compared to the rabbit. Their value, however, essentially corresponds to our mean and is in a sense determined in much the same way. Finally, the range of our values includes the values of 10 and 6 days for the half life of serum proteins in man and the rat, respectively, as cited by Sprinson and Rittenberg (1949).

Our low half life values around 3 days approximate the time in which the major portion of horse antitoxin disappeared when passively transferred to rabbits as reported by Glenny and Hopkins (1922). These authors found the rate of antibody decline to be triphasic in their rabbits. The first and third phases consisted of steep declines interrupted by a second phase with a slower decline. The first phase lasted only about a day. The second one, which they considered to be due to the natural elimination of foreign protein, lasted about a week. It was clearly semilogarithmic in character with a half life of about 2.25 to 2.5 days. If this short half life is due in part to the use of foreign serum, our low values may be referable to the operation of some mechanism other than metabolic degradation. We have no evidence on this point at present.

The importance of the spleen is shown in another way by Jacobson et al (1949). These authors found that the capacity to form hemolysin was almost completely suppressed in rabbits receiving a total body X irradiation of 800 r, but was largely retained when the spleen was shielded with lead during irradiation. They also found that shielding the appendix protected the hemolysin-producing capacity, whereas we observed no decrease in three rabbits after appendectomy. This divergence of findings probably indicates interactions among the organs for which we have no data at present. Interaction between the organs may also be indicated by the apparently shorter antibody half life for our operated controls (6.5 days) which is significantly lower statistically than that in intact animals receiving the same dose ($P<0.01$) but is probably higher than that in rabbits receiving the smaller doses (P about 0.02).

Closely connected with the question of the cessation of antibody formation by the spleen is the exact time and abruptness of this cessation. In curves such as shown in figure 3A which were commonly obtained with the small and sometimes the larger amounts of antigen, antibody accumulates in the serum at a single constant rate, and there seems little doubt that the spleen ceases to produce antibody at the peak titer. Similarly, the second phase with a smaller value of k is also probably the result of splenic activity because more antibody is formed during this interval than is attained in most rabbits splenectomized near the time of immunization. That the secondary rise may be due to nonsplenic sources, in a few animals, however, is shown by the high titers in two rabbits splenectomized on the day of immunization (fig. 5C) and by the rise in antibody after splenectomy on the 6th (fig. 6A and 6B) or 8th day in 4 of 10 rabbits (table 9). It is difficult to decide when the spleen ceases to function when there is a third phase consisting of a plateau (fig. 3C) or a slight decline of several days (fig. 4B). The amount of antibody necessary to maintain such a high titer is close to amounts usually observed in animals splenectomized near the time of immunization. In addition, the fact that nonsplenic sources can occasionally form such amounts of antibody is demonstrated in figures 5C and 6A.

The abruptness with which the spleen ceases to produce antibody cannot be decided with certainty, but it seems to

occur within a period of less than one day in many rabbits. Thus, when daily and especially semi-daily observations were plotted semilogarithmically from rabbits with a single rate of increase and decline, the peak titer was preceded by a hemolysin titer in line with preceding ones for 2 to 4 days and was followed by one in line with succeeding ones. The same was true for rabbits with 2 rates of increase and a decline. In these rabbits the point just preceding the peak was in line with the second slower rate of increase which generally lasted for 2 to 3 days (fig. 2). It should be noted that an antibody decline frequently occurred in splenectomized rabbits which was similar in rate to that in intact animals but was shorter (fig. 5B).

The virtual cessation of antibody production by the spleen early in the process of immunization could be either the result of inhibitory substances or of the exhaustion of some necessary material in antibody synthesis. The apparent abruptness of the termination favors the latter hypothesis because the first would probably be associated with the gradual accumulation of inhibitory substances, and therefore, with a gradual lowering of antibody production. Among such necessary materials might be the frequently postulated antigen templates. Preliminary experiments indicate, however, that the spleen ceases to function in the midst of continuing daily intravenous injections of antigen. No matter what the explanation, the early cessation of splenic function and the continued activity of nonsplenic sources may reasonably be associated with the much higher initial activity of the spleen. The previous discussion relates to the termination of splenic activity. Even more difficult to explain are the abrupt changes in the rate of antibody production by the spleen as indicated by two distinct rates of increase before the peak titer in many rabbits.

Actual release of antibody from the spleen must be extremely rapid as we never found large accumulations of antibody in the spleen. Thus, in work which we shall publish later in detail, we found that the splenic content of hemolysin per unit weight was never greater than that of the serum except during the first few days after immunization when titers were low, titrations somewhat unreliable and absolute differences small. Peak titers occurred in the spleen from the 6th to the 8th day. They varied over a wide range and were <1 to 50% of serum titers. Thereafter, the titers were rarely over 20% of the serum titers per unit volume. The small amount of hemolysin recovered from the spleen and the small size of the spleen indicate very little antibody activity within the splenic cells.

Koshland and Burrows (1950) studied the rise and fall of fecal antibody in guinea pigs immunized with cholera O vaccine. This antibody is probably not derived from serum (Burrows et al, 1947 and 1948), and they assume that when liberated into the intestine it is excreted as soon as it is produced, whereas, when liberated into the serum, it accumulates. They, therefore, concluded that the change in fecal antibody is a measure of the rate of antibody production. Their data points, although few, indicate that high fecal titers were largely limited to a 6-day period beginning on the third day after a single injection of antigen. This finding is in line with our conception of a sharply limited splenic activity although the site of formation is probably different.

The antibody curves in intact and in some splenectomized animals receiving 1 ml 10% cells resemble, respectively, the two types of response of guinea pigs to diphtheria formol-toxoid found by Holt (1949), although the route of immunization and the time intervals are different and the basic mechanisms may be different. Holt found that the

antitoxin curve following a single injection of formol-toxoid is followed by two types of response: (1) a relatively high titer attained in 6 to 7 weeks followed by a decline and a plateau which persisted more or less unchanged for an extended period (see earlier work of Glenny and Südmerson, 1921, on guinea pigs; and Barr and Glenny, 1947, on horses), and (2) a rise in titer which reached a peak in about 4 weeks and remained at about the same level over a long period similar to the plateau noted in the first type. The second type of response, the author considers to be the theoretical pure primary response, i.e., a sudden rise of antitoxin between the 2nd and 4th weeks which is maintained at its maximum value for an extended period. The high temporary rise and fall in the first type, he believes, to be due probably to traces of toxin in the toxoid (cf. Glenny et al, 1925) which become locally fixed in the nonimmune animal and later, after being released, act as a secondary antigenic stimulus. A parallel situation was found when optimum combinations of toxoid and aluminum phosphate carrier were given subcutaneously. When the nodules resulting from the immunizing injection were left in the animal or were excised after 14 days, the antibody curve showed the initial rise to a high peak with a rapid fall to the continuing plateau level. When the nodules were excised 7 to 10 days after injection, the resulting antibody curve lacked the high peak and initial rapid fall but was maintained at a plateau of the same titer as the plateau after the rapid fall in animals in which the nodules were not excised or were excised after 14 days.

This work raises the question as to whether our peaks of splenic antibody could be an expression of the fixation of antigen with subsequent release to give what is essentially a secondary response at the end of the true primary rise of antibody. Such a mechanism seems unlikely with the antigen we used and the manner in which it was injected. Furthermore, rabbits 80, 83 and 84 were splenectomized 3 to 4 hours after the antigen was injected. At this time, most of the antigen normally taken up by the spleen should have been fixed by it. One of these rabbits showed the typical low titers usually obtained. Two, however, showed not only the highest titers encountered in animals splenectomized early in immunization but also changes in the value of k usually found in intact animals receiving this dose of antigen.

Burnet and Fenner (1949), in reviewing the literature and the work of their own group, consider a logarithmic rise of antibody as characteristic of the secondary antibody response, but not of the primary response. Although they believe this conclusion is chiefly true for toxins and toxoids and is not as evident for particulate microbic antigens, the question might be raised as to whether we might unwittingly be dealing with a secondary response in our rabbits, especially as our antigen is in part a Forssman antigen which may be ingested in food or acquired by infection. Such a possibility seems unlikely because in those animals which showed a normal hemolysin titer of $1:<1$ (i.e. >1 ml of undiluted serum did not hemolyze 1 ml 2% red cells), there was just as rapid a semilogarithmic increase in antibody as in animals with a normal hemolysin titer around 1:100. Furthermore, with the small amount of cells, the antibody titer varied from 1:80 to 1:200 three months after injection. In addition, we have been unable to find many papers dealing with relatively quantitative methods, in which closely spaced determinations were taken during the antibody rise. Monomolecular types of curves, may, therefore, be more common than suspected. In any case their significance is important. They suggest, as Burnet and Fenner points out for the secondary

response, a "multiplication of some entity concerned with antibody production." The constant of increment in our rabbits is remarkably high for the initial rise with a modal point around 1.5 and an extreme value of 3.3.

Many published graphs of antibody decline simulate a bimolecular type of curve, as suggested by Jensen (1933) in his study of the disappearance of antitoxin in children following a single subcutaneous injection of concentrated anatoxin. Such curves indicate a decreasing rate of antibody formation superimposed on the logarithmic decay. Our monomolecular type of decline probably results from the fact that the spleen produces a large fraction of the initial antibody and then abruptly stops. Other sites may react similarly, as indicated by the initial decline in some splenectomized animals. Here, as in the case of the antibody rise, more work with closely spaced observations on the same animal is necessary to ascertain whether or not the situation we studied is unique.

We have found no correlation of the three different amounts of antigen injected within an 80-fold range from 2×10^7 to 160×10^7 red cells with the peak antibody titer, the initial value of k, or the antibody titer at the end of the first phase of antibody rise. We did, however, find that the amount of antigen injected was positively correlated with the number of phases (whether 1, 2 or 3 as illustrated in figure 3) of the initial antibody rise ($r=0.38$ and $P<0.01$) (cf. McLean et al, 1945).

The results of splenectomy are of interest per se aside from demonstrating the importance of the spleen in the initial phase of antibody formation. As pointed out above, the peak titer was markedly low in most animals splenectomized on the day of immunization through the 4th day, was variable in animals splenectomized on the 5th day, and was practically the same as in intact animals when splenectomy was performed on the 6th and 8th days. The last result was to be expected because peak hemolysin titers had already been reached on the 8th day and occasionally on the 6th day. It is, however, striking that 2 rabbits splenectomized 14 days before and 1 of 3 rabbits splenectomized 28 days before immunization showed a marked reduction of peak titer. Three rabbits splenectomized 98 days before immunization behaved as do intact animals. The last finding is in contrast to those of Rowley (1950), who reported that rats splenectomized 6 months before immunization behave as do those splenectomized at about the time of immunization.

The entire antibody curve is unquestionably the resultant of the rate of synthesis and of antibody destruction with the two processes continuing as long as active immunity persists (Heidelberger et al, 1942). This is excellently shown in an experiment of Schoenheimer et al (1942), who fed isotopically labelled glycine to rabbits from the 10th to the 13th day after the final injection of pneumococci when antibody was declining. The total antibody nitrogen determinations fit a semilogarithmic curve and indicate an apparent half life of about 12 days, i.e., a value near the range of those reported in the present work. Nevertheless, the N^{15} content of antibody rose rapidly during the feeding of glycine labelled with N^{15} while the antibody was decreasing. Antibody, therefore, was being synthesized and decaying simultaneously. If conclusions reached from our results can be transferred to immunization against pneumococcus, they may indicate that their antibody decline involved splenic antibody and the synthesis involved nonsplenic sources.

Finally, the present work indicates that after a single intravenous injection of red cells in the amounts we used, the spleen forms a relatively large amount

of antibody within a brief, restricted period, whereas other antibody-producing units form antibody at a slower rate over an extended period and maintain the serum antibody at a relatively low level. Total production of nonsplenic sources may be larger than that of the spleen. The frequent occurrence of successive antibody declines suggests that a series of sites may successively cease to produce antibody rather than that all lower their production gradually.

CONCLUSIONS

1. The rise and decline of hemolysin in rabbits following a single intravenous injection of sheep red cells fit a series of discontinuous curves described by the "compound interest" or monomolecular reaction equation in which the constant of increment differs.

2. Splenectomy significantly depressed antibody formation (to about 20% on the average) in 12 of 14 rabbits when performed on the day of immunization through the 4th day. It was occasionally effective on the 5th day and was practically ineffective on the 6th and 8th days. Splenectomy 14 and 28 days before immunization was effective in 2 and in 1 of 3 rabbits, respectively, and was ineffective in 3 rabbits operated on 98 days before immunization.

3. In 54 intact animals, the antibody rise consisted of one to three phases. The initial phase showed a high constant of increment, which sometimes abruptly changed to a second phase with a lower constant of increment. The third phase approximated a plateau. Increasing the amount of red cells was correlated with an increase in the number of phases, but not with peak titer or the magnitude of the constants of increment.

4. The initial decline in animals given 1.25 ml 0.1% or 1% red cells tended to be rapid with an antibody half life of 5.5 ± 0.3 days, sometimes followed by a slower decline, until a level was reached which was about the level characteristic of splenectomized animals. The initial decline in those given 1 ml 10% red cells was slower (indicated half life of 7.95 ± 0.4 days) probably because of a higher activity of nonsplenic sources.

5. Comparison of the antibody curves in intact rabbits and rabbits splenectomized about the time of immunization indicates that the spleen forms most of the antibody during the initial rise and abruptly stops forming antibody at or about peak antibody titer. Splenic antibody then disappears due to normal metabolic degradation until the continuing level of antibody from nonsplenic sources is reached. Nonsplenic sources form antibody at a rate only slightly less than the rate of antibody decay over a long period and probably produce more total antibody than the spleen. Although the entire antibody curve is the result of the continuing synthesis and metabolic decay of antibody, the high initial titer results largely from the spleen and the continuing synthesis from nonsplenic sources.

6. Antibody is released from the spleen shortly after it is recognizable as antibody as indicated by the fact that antibody titer of the spleen was rarely over 20% of that of the serum except during the first few days when titers were low and determinations somewhat unreliable.

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IN VITRO SENSITIVITY OF PATHOGENIC ENTERIC BACTERIA TO VARIOUS ANTIBIOTICS

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The present study is part of a broader one in which an attempt is made to derive what might be called an antibiotic spectrum for each of the common species of human pathogenic bacteria. Each of these spectrums is constructed from the results of in vitro sensitivity tests carried out by uniform methods on strains derived from various sources and tested with the most widely used antibiotics and with some others which appear to be of particular interest. This paper deals primarily with the results obtained with human enteric pathogens that were tested with seven antibiotics. When these tests were completed, two additional antibiotics which seemed worthy of study became available, and a few selected strains were also tested with these new antibiotics.

MATERIALS AND METHODS

Strains tested.—About one-third of the strains included in this study were isolated from the stools or blood of patients acutely ill with enteric infections at the Boston City Hospital or at neighboring hospitals. They were obtained in pure culture and identified in a preliminary manner either in the Bacteriological Laboratory of the Mallory Institute of Pathology by Miss Marion E. Lamb or in the Diagnostic Laboratory of the Massachusetts Department of Public Health through the courtesy of Dr. Robert A. MacCready. Phage typing of all strains of *Salmonella typhosa* and final classification of the strains of the other salmonellas and of the shigellas were done in the Enteric Bacteriology Laboratory of the Communicable Disease Center, U. S. Public Health Service, Chamblee, Georgia, through the courtesy of Dr. P. R. Edwards and Dr. W. H. Ewing. The remaining strains, which were generously provided by Dr. Edwards and Dr. Ewing, included subcultures of strains of various phage types of *S. typhosa* and of representative strains of the different types of other salmonella and shigella species which they had received from various widely scattered parts of this country.

Antibiotics.—The following antibiotic preparations were used in tests with all of the strains and were the same as those used in similar studies with other organisms:[1]

Crystalline penicillin G, potassium salt, 1595 units per mg

Streptomycin calcium chloride complex (Merck)

Bacitracin, 34 units per mg*

Polymyxin (Polymyxin D) hydrochloride (Lederle B71)†

Aerosporin (Polymyxin B) hydrochloride‡

Crystalline chloromycetin (chloramphenicol)§

Crystalline aureomycin hydrochloride†

The antibiotics which became available to us recently and were used for tests with a few of the strains were:

Neomycin sulfate, 173 units per mg*

Crystalline terramycin hydrochloride‡

Concentrations of these antibiotics were all expressed in μg of the active agent per ml of medium.

Tests for sensitivity.—The choice of the method used throughout this study was based on many

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1. Frank, P. F., Wilcox, C. and Finland, M. 1950, *In vitro* sensitivity of coliform bacilli to seven antibiotics (penicillin, streptomycin, bacitracin, polymyxin, aerosporin, aureomycin and chloromycetin). J. Lab. & Clin. Med. **35**: 188–204.

* Supplied by Dr. Lawrence W. Smith of Commercial Solvents Corporation.

† Supplied by Dr. Stanton M. Hardy of Lederle Laboratories.

‡ Supplied by Dr. Gladys L. Hobby of Chas. Pfizer & Co.

§ Supplied from Dr. E. A. Sharp of Parke, Davis & Co.

TABLE 1.—*Results of simultaneous in vitro tests for sensitivity of selected pathogenic strains of* Salmonella *and* Shigella *to seven antibiotics.*

No.	Organism	Penicillin		Streptomycin		Bacitracin		Polymyxin		Aerosporin		Aureomycin		Chloromycetin	
		P	C	P	C	P	C	P	C	P	C	P	C	P	C
1	*S. typhosa*, A*	1.9	7.5	25.0	100.0	3,000	12,000	1.6	3.1	1.6	1.6	3.1	25.0	6.3	6.3
2	*S. typhosa*, B_1	1.9	3.8	3.1	25.0	3,000	6,000	0.8	0.8	1.6	1.6	6.3	25.0	6.3	6.3
3	*S. typhosa*, C	1.9	7.5	6.3	50.0	6,000	12,000	1.6	1.6	1.6	1.6	3.1	50.0	6.3	6.3
4	*S. typhosa*, E_1	3.8	15.0	25.0	50.0	6,000	12,000	1.6	1.6	1.6	1.6	3.1	25.0	3.1	3.1
5	*S. typhosa*, F_3	1.9	3.8	6.3	50.0	6,000	12,000	1.6	1.6	1.6	1.6	3.1	25.0	6.3	6.3
6	*S. typhosa*, G	1.9	3.8	6.3	25.0	6,000	6,000	0.4	1.6	1.6	3.1	3.1	25.0	6.3	6.3
7	*S. typhosa*, K	3.8	7.5	12.5	50.0	6,000	6,000	0.8	1.6	1.6	3.1	3.1	25.0	6.3	12.5
8	*S. typhosa*, L_1	3.8	7.5	6.3	25.0	6,000	6,000	0.8	0.8	1.6	3.1	6.3	25.0	6.3	6.3
9	*S. typhosa*, T	1.9	3.8	25.0	50.0	6,000	12,000	0.8	1.6	1.6	3.1	3.1	25.0	12.5	12.5
10	*S. typhosa*, W	1.9	15.0	25.0	100.0	6,000	12,000	1.6	1.6	1.6	1.6	3.1	25.0	6.3	6.3
11	*S. enteritidis*	7.5	15.0	12.5	25:0	12,000	12,000	1.6	1.6	1.6	6.3	6.3	50.0	6.3	6.3
12	*S. newington*	15.0	30.0	12.5	200.0	12,000	24,000	1.6	3.1	1.6	1.6	6.3	25.0	6.3	12.5
13	*S. paratyphi B*	7.5	15.0	25.0	100.0	12,000	12,000	6.3	25.0	3.1	6.3	12.5	25.0	6.3	12.5
14	*S. newport*	15.0	30.0	6.3	100.0	12,000	12,000	3.1	3.1	3.1	3.1	3.1	25.0	6.3	12.5
15	*S. swipeslifer*	7.5	15.0	25.0	200.0	24,000	24,000	3.1	3.1	0.8	1.6	3.1	25.0	12.5	12.5
16	*S. typhimurium*	15.0	60.0	25.0	200.0	6,000	6,000	1.6	3.1	3.1	3.1	3.1	25.0	12.5	12.5
17	*S. mississippi*	7.5	15.0	25.0	100.0	12,000	12,000	1.6	1.6	1.6	1.6	6.3	25.0	3.1	6.3
18	*S. pullorum* XII_3	3.8	3.8	12.5	25.0	6,000	6,000	0.2	0.2	0.2	0.8	1.6	12.5	0.8	1.6
19	*S. paratyphi A*	15.0	30.0	12.5	100.0	12,000	12,000	0.8	0.8	0.8	0.8	12.5	50.0	6.3	12.5
20	*S. tennessee*	7.5	7.5	12.5	200.0	12,000	12,000	1.6	1.6	1.6	1.6	6.3	50.0	6.3	12.5
21	*Shig. sonnei*	30.0	30.0	12.5	25.0	3,000	3,000	0.4	0.8	3.1	6.3	12.5	50.0	3.1	12.5
22	*Shig. sonnei II*	120.0	240.0	12.5	50.0	6,000	6,000	0.8	0.8	0.8	0.8	3.1	50.0	6.3	6.3
23	*Shig. flexneri Ia*	60.0	120.0	6.3	12.5	6,000	12,000	0.8	0.8	0.8	1.6	3.1	25.0	0.8	1.6
24	*Shig. flexneri VI*	15.0	30.0	12.5	100.0	6,000	12,000	0.8	1.6	0.4	0.8	3.1	25.0	3.1	3.1
25	*Shig. alkalescens*	60.0	60.0	6.3	12.5	6,000	12,000	0.8	0.8	0.8	3.1	1.6	12.5	3.1	6.3
26	*Shig. dysenteriae II*	7.5	15.0	25.0	50.0	6,000	6,000	0.4	0.8	0.8	0.8	3.1	25.0	0.8	1.6
27	*Shig. dysenteriae V*	30.0	60.0	12.5	200.0	3,000	6,000	0.8	1.6	0.8	0.8	3.1	50.0	6.3	12.5
28	*Shig. boydii 1*	60.0	60.0	12.5	100.0	12,000	12,000	0.8	1.6	0.8	1.6	6.3	50.0	6.3	12.5
29	*Shig. boydii V*	15.0	15.0	3.1	25.0	12,000	12,000	0.4	0.8	0.8	0.8	3.1	12.5	0.8	3.1
30	*Shig. dispar IIa*	120.0	240.0	6.3	25.0	6,000	6,000	0.8	1.6	1.6	3.1	6.3	50.0	6.3	6.3

* The designations of the strains of *S. typhosa* indicate the phage types as determined at the Communicable Disease Center, U.S.P.H.S., Chamblee, Ga., courtesy of Dr. P. R. Edwards who also supplied strains 2, 3, 5, 6, 7, 8 and 17 to 20, inclusive. Strains 22 to 30, inclusive, were furnished by Dr. W. H. Ewing of the Shigella Unit. Strains 1, 4, 9, 10 and 21 were isolated at the Boston City Hospital.

P = minimum concentration which produced marked inhibition in 24 hours; C = minimum concentration giving no discernible growth at 48 hours.

All values in μg/ml.

comparative tests with different methods which indicated that wide variation in the values for sensitivity, ranging up to 128-fold or even more, could frequently be obtained with the same strain and the same antibiotic depending on the details of the method employed.[2] The simple plate-dilution method which was chosen gives the highest values as compared with any of the methods generally used, but it has proved to be the most convenient one for large numbers of tests such as were carried out in this study. It also proved to be the most suitable method when different antibiotics are to be compared.

Serial twofold dilutions of each antibiotic in the appropriate range were prepared in plates of heart infusion agar (Difco), pH 7.2 ±. The dilutions of all of the antibiotics except chloromycetin were freshly prepared from the dry preparation at the time of each test. A standard solution of chloromycetin was kept in the frozen state at −25 C and used for all of the tests. Because of the tendency of aureomycin to deteriorate rapidly in neutral or alkaline solution, special care was taken to make up the dilutions of this antibiotic immediately before the plates were poured, and the cultures were streaked on them promptly after they had solidified. Each plate was divided into segments, and one segment of each plate was streaked with a 1 mm loopful (containing approximately 10^7 organisms) of a fresh, fully grown broth culture of one of the strains to be tested. The plates were then examined for growth in a good light with a magnifying lens (3×) after 24 hours and again after 48 hours of incubation at 37 C. Groups of 10 to 25 strains were tested simultaneously with the 7 antibiotics that were available throughout the study, and the tests with neomycin and terramycin were carried out separately at a later date. Standard laboratory strains (*Klebsiella pneumoniae*, strain T, and *Streptococcus* 98) were included in each test.

The sensitivity in each instance was defined as the minimum concentration of antibiotic, in μg per ml, on which there was complete inhibition of growth, that is, no evidence of any growth was discernible after 48 hours. The value for partial inhibition was taken as the minimum concentration on which there was marked inhibition of growth at 24 hours as evidenced by the appearance of either very minute colonies or of only a moderate number of distinct colonies.

RESULTS

A total of 164 strains were tested with 7 antibiotics, and 11 of the strains were tested, in addition, with neomycin and terramycin. The strains of *S. typhosa*, of which there were 35, included representatives of 15 phage types; there were 55 strains of other salmonellas which included 1 or more specimens of 22 types that were identified serologically and also of 2 unidentified types, while 36 different types and subtypes were included among the 74 strains of shigellas.

The results of the tests with 30 representative strains are listed in table 1. Both the partial and complete inhibiting concentration for each of 7 antibiotics are given in μg of the active agent per ml of medium in this table. The greatest variations in the complete inhibiting concentrations were noted for all of the strains among the different antibiotics. Thus, all of the strains were most sensitive to polymyxin and aerosporin and were about equally sensitive to these two agents. Chloromycetin ranked next, although penicillin was about equally effective against most of the salmonellas, particularly the strains of *S. typhosa*. Aureomycin ranked next and was followed by streptomycin, while penicillin was about as active as the latter against the shigellas. Bacitracin was by far the least active of all of these antibiotics.

Except in this general way, there did not seem to be any definite correlation between the sensitivity of any given strain to one antibiotic and its sensitivity to any of the others. Nor was there any correlation between the type and the sensitivity. The strain of *Salmonella pullorum* (No. 18) may be an exception; it was more sensitive than the others to all of the antibiotics except bacitracin. Three other strains of *S. pullorum* were also among the most sensitive to the same antibiotics. There were only moderate variations in the sensitivity of different strains to the same antibiotic.

2. Jackson, G. G. and Finland, M. Unpublished observations.

The results of sensitivity tests carried out at different times with the control strains indicated up to 4-fold variations with penicillin and streptomycin. Only a 2-fold difference was observed in tests done at different times with chloromycetin, polymyxin, aerosporin and bacitracin, while the results obtained with aureomycin varied as much as 8-fold from one test to another.

The difference between the partial and complete inhibiting concentrations, which indicates the sharpness of the end point of the tests, varied among the different antibiotics and among the different strains tested with the same antibiotic. Here again, the greatest variations were observed among the different antibiotics. Thus, the sharpest end points were obtained with polymyxin, aerosporin and bacitracin. With the great majority of the strains the partial and complete inhibiting concentrations of these three antibiotics were either the same or showed only a 2-fold difference, that is, either the next concentration beyond the one which produced complete inhibition showed full growth or only that concentration produced partial inhibition. With chloromycetin and penicillin, partial inhibition was usually observed in one or two dilutions, that is, the ratio of complete to partial inhibiting concentrations was most often 2 or 4. With aureomycin and streptomycin, on the other hand, the range of partial inhibition was greatest, and the ratio of complete to partial inhibiting concentrations was usually 4 or 8, and occasionally it was even greater.

From the results of the tests done on all of the strains, curves were constructed to represent the distribution of the sensitivities of all of the strains of *S. typhosa* for each of the antibiotics, and the same was done for the other salmonellas and for the shigellas. For convenience in making comparisons, the cumulative percent of strains was used as the ordinate, and the minimum complete inhibiting concentrations in μg per ml were used for the abscissa. The resulting curves, which constitute what might be called the antibiotic spectrums of the enteric group of organisms, are shown in figure 1. In this figure each antibiotic is represented by a curve or band in the spectrum of the bacterial species (or genus). The steepness of each curve indicates the uniformity of the results of the sensitivity tests done with different strains and the same antibiotic. The in vitro activity of the different antibiotics may be readily compared by their position from left to right.

The ranges of concentration of these 7 antibiotics which inhibit the great majority of the strains are listed, for convenience, in table 2. From this table and from figure 1 these antibiotics can readily be arranged in the order of their activity against the different enteric

TABLE 2.—*Range of concentrations of 7 antibiotics which completely inhibit more than 75% of strains of enteric pathogens.*

Antibiotic	*S. typhosa* (35 strains)		*Salmonella* except *typhosa* (55 strains)		*Shigella* (74 strains)	
	μg/ml	Percent of strains	μg/ml	Percent of strains	μg/ml	Percent of strains
Penicillin	3.8–15	95	15–30	87	30–120	82
Bacitracin	6–12 mg	92	12–24 mg	87	6–12 mg	87
Polymyxin	0.8–1.6	88	0.8–3.1	93	0.8–1.6	85
Aerosporin	0.8–3.1	97	1.6–6.3	86	0.8–6.3	93
Streptomycin	25–200	94	25–200	97	6.3–100	96
Aureomycin	12.5–25	91	25–50	90	12.5–50	95
Chloromycetin	3.1–12.5	100	6.3–12.5	92	3.1–12.5	86

pathogens. Against *S. typhosa* they rank as follows: (1) polymyxin, (2) aerosporin, (3) chloromycetin, (4) penicillin, (5) aureomycin, (6) streptomycin and (7) bacitracin. The same order obtains for the other salmonellas, but, while chloromycetin and penicillin are about equally active against *S. typhosa*, the former agent is appreciably more active against the other salmonellas. For the shigellas the order of activity of these 7 antibiotics is: (1) polymyxin, (2) aerosporin, (3) chloromycetin, (4) streptomycin, (5) aureomycin, (6) penicillin and (7) bacitracin. The relative position of streptomycin and aureomycin in this instance is arbitrary since a similar proportion of the strains were slightly more sensitive to each of these agents.

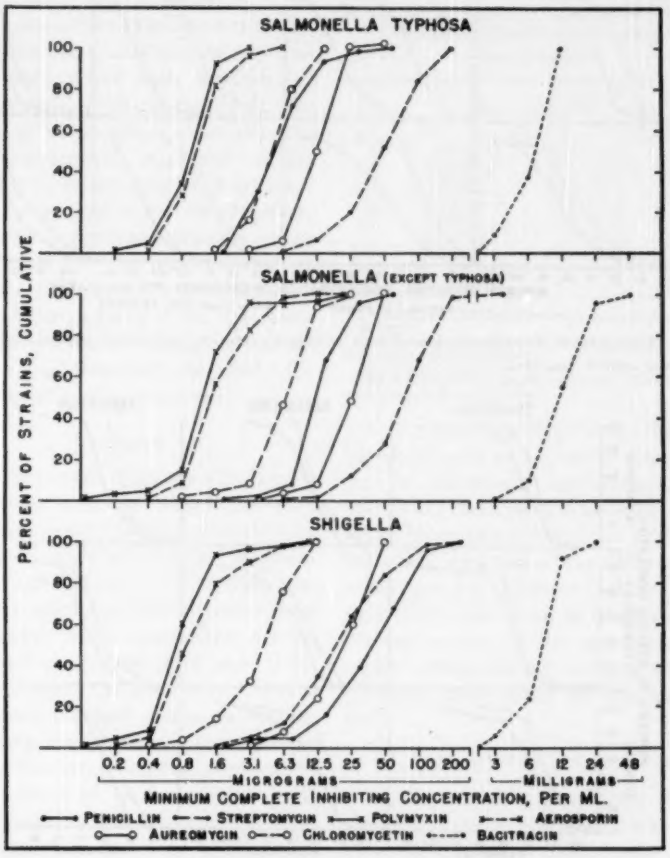

FIG. 1.—Antibiotic spectrums of enteric bacteria.

Comparisons of the partial and complete inhibiting concentrations for all of the strains of each organism and for the different antibiotics are shown in figures 2 and 3. The wide separation of the aureomycin and streptomycin curves in figure 2 reflects the wide range of

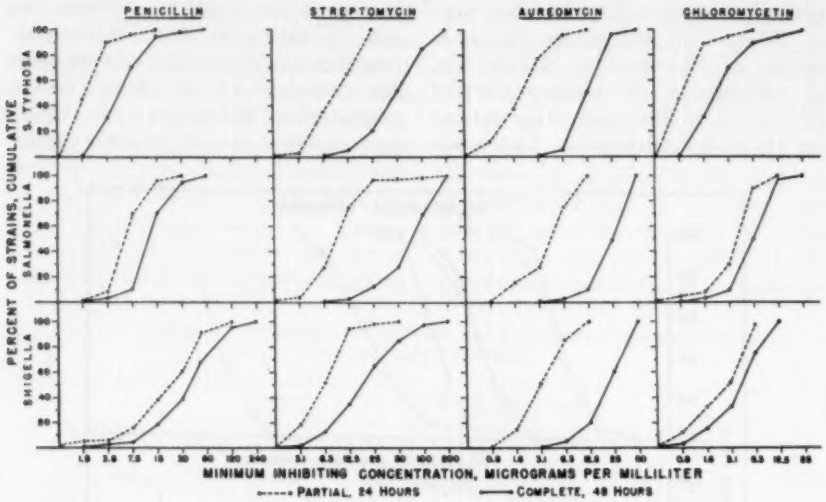

FIG. 2.—Comparison of concentrations of four antibiotics required to produce partial and complete inhibition of enteric bacteria.

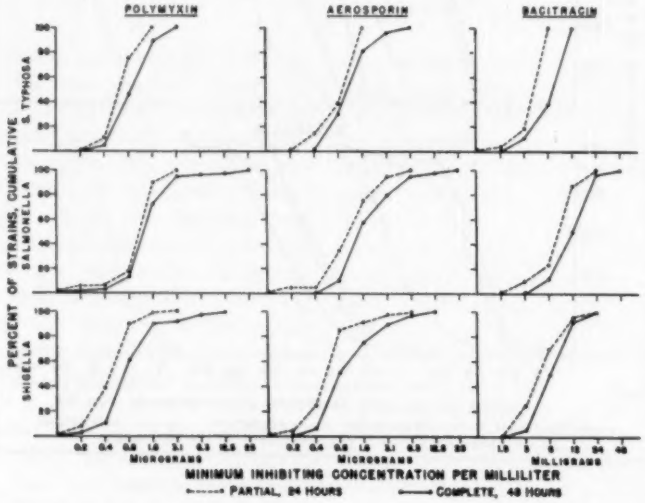

FIG. 3.—Comparison of concentrations of three additional antibiotics which produce partial and complete inhibition of enteric bacteria.

partial inhibition of all of the organisms by these agents. The close proximity of the curves from polymyxin, aerosporin and bacitracin in figure 3 reflects the sharp end points obtained with these antibiotics. The curves for penicillin and chloromycetin in figure 2 show an intermediate range of partial inhibition.

The new antibiotics, neomycin and terramycin, became available to us after these studies were completed. Only a representative group of strains was therefore tested with these two agents and the results are shown in table 3. As far as could be judged from these results, neomycin appeared to be slightly more active than streptomycin, on a weight basis, and terramycin was slightly more active than aureomycin. On a unit basis, however, neomycin was appreciably more active than streptomycin. Terramycin appeared to be about equally active against *S. typhosa*, the other salmonellas and the shigellas, while there was some indication that neomycin may be more active against *S. typhosa* than against the other organisms. With both of these new antibiotics there was a wide range of partial inhibition similar to that observed with streptomycin and aureomycin.

TABLE 3.—*Sensitivity of representative strains of* Salmonella *and* Shigella *to neomycin and terramycin.*

No.	Organism	Neomycin		Terramycin	
		P	C	P	C
2	*S. typhosa*, B_1	2.3	18.0	1.6	12.5
3	*S. typhosa*, C	2.3	18.0	0.8	12.5
31	*S. typhosa* F_1	4.5	36.0	0.8	12.5
11	*S. enteritidis*	9.0	72.0	1.6	12.5
13	*S. paratyphi B*	4.5	72.0	1.6	12.5
14	*S. newport*	9.0	72.0	1.6	12.5
16	*S. typhimurium*	9.0	72.0	1.6	12.5
21	*Shig. sonnei*	9.0	72.0	0.4	6.3
28	*Shig. boydii I*	18.0	36.0	0.4	12.5
32	*Shig. dysenteriae I*	4.5	18.0	0.4	12.5
33	*Shig. flexneri I*	4.5	72.0	0.8	25.0

P = minimum concentration producing marked inhibition in 24 hours.
C = minimum concentration on which there was no discernible growth after 48 hours.
All values are expressed in μg/ml.
The numbers of the strains, except 31, 32 and 33, correspond to those shown in table 1.

COMMENT

It is well known that many factors in the in vitro tests for sensitivity may profoundly influence the quantitative results and that these factors vary from one antibiotic to another. The data presented are valid only for the conditions under which these tests were carried out. These conditions were quite uniform throughout the present studies and thus permit the best comparisons that are possible with agents of such diverse activity. The interpretation of these results in terms of clinical effectiveness likewise requires considerable caution. Many factors in the host such as absorption, excretion, diffusion and plasma binding and some of the characteristics of the infection itself, such as the intracellular location of the organisms, also affect the activity of the different agents against various diseases in diverse ways. Moreover, the toxicity of the compounds alone may be the determining factor in its usefulness as is well exemplified in polymyxin and aerosporin. These agents are by far the most active in vitro against all of the strains, but they are nevertheless too toxic for general use in the treatment of these infections.

The present findings may, therefore, be useful only as a general approximation of the relative usefulness of these agents in therapy. Because of the diverse biochemical activities and metabolic requirements of the different bacterial species, however, these results may hold some clues or hints as to the diverse nature of the mechanisms by which antibiotics of varying chemical structure exert their antimicrobial action.

SUMMARY AND CONCLUSIONS

In vitro tests for sensitivity to seven antibiotics were done on a wide selection of strains of pathogenic enteric bacteria.

These organisms as a group varied markedly in their sensitivity to the different antibiotics. Different strains of the same species, however, varied only slightly in their sensitivity to any single antibiotic.

The range of partial inhibition also

varied chiefly with the antibiotic, being widest for aureomycin and streptomycin and less so for penicillin and chloromycetin, whereas the tests with polymyxin, aerosporin and bacitracin yielded fairly sharp end points.

On the basis of the results of these tests, cumulative distribution curves of the sensitivity of the strains to the various antibiotics were constructed which together constitute what may be considered antibiotic spectrums for *Salmonella typhosa*, for other types of salmonellas and for the shigellas.

From these spectrums the order of activity of the different antibiotics was readily discernible. For *S. typhosa*, as well as for the other salmonellas, the order of diminishing activity on a weight basis was: (1) polymyxin, (2) aerosporin, (3) chloromycetin, (4) penicillin, (5) aureomycin, (6) streptomycin and (7) bacitracin. For the shigellas this order was: (1) polymyxin, (2) aerosporin, (3) chloromycetin, (4) streptomycin, (5) aureomycin, (6) penicillin and (7) bacitracin.

Polymyxin and aerosporin were about equally active against each of the groups of bacteria. Penicillin was about as active as chloromycetin against *S. typhosa* but was appreciably less active against the other salmonellas and the shigellas. Streptomycin and aureomycin were about equally active against the shigellas.

A few selected strains were also tested for sensitivity to neomycin and terramycin. Neomycin appeared to be slightly more active than streptomycin, while the activity of terramycin was intermediate between that of chloromycetin and that of aureomycin. Wide ranges of partial inhibition were observed in the tests with both of these new antibiotics.

COMPARISON OF THE ERYTHROCYTE AND LEUKOCYTE CELL SURFACES OF GUINEA PIGS BY USE OF AGGLUTINATION TECHNIQUES

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It is clear that a knowledge of the physical and chemical composition of the walls of phagocytic cells is essential to an understanding of the phenomenon of phagocytosis. It is not easy to obtain large quantities of leukocytes for chemical analyses. Furthermore, it is probable that the composition of the cell wall is so complex that the process of fractionation would destroy important elements in its physical and chemical make-up. Therefore another approach to this problem was adopted for work reported in this paper. It was decided to use various biological materials which were known to act on erythrocytes to produce results such as agglutinations. In some cases, as, for example, the action of the influenza virus in agglutinating red blood cells, there is evidence of the possible chemical nature of the site of action of the virus.[1] Thus, if the influenza virus agglutinated both the erythrocyte and the leukocyte of a given host, it might be assumed, at least tentatively, that there was a common or similar component in the walls of both cells. Also, immunological studies were made comparing red blood cell membrane and leukocyte surface in the hope that demonstration of differences or similarities would give indirect evidence of the surface configuration of the leukocytes in terms of what we know of the chemistry of the red blood cell membrane.[2] For similar reasons the relative agglutinating effect of pneumococcus polysaccharide and navy bean extracts on guinea pig leukocytes and erythrocytes has been studied.

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1. de Burgh, P. M., Yu, P. C., Howe, C. and Bovarnick, M. 1948, J. Exper. Med. **87**: 1–9.
2. Ponder, E. 1947, Ann. N. Y. Acad. Sc. **48**: 577–704.
3. Nungester, W. J. and Ames, A. M. 1948, J. Infect. Dis. **43**: 50–54.

MATERIALS AND METHODS

Erythrocyte suspensions.—Guinea pig blood was obtained by cardiac puncture usually without anesthesia. Ether anesthetic was not used in these experiments because of the possibility that it would produce changes in cell surfaces. When an anesthetic was needed, nitrogen gas was used, with quite satisfactory results as a substitute for ether. The blood was placed in 50 ml centrifuge tubes containing 3 to 4 ml of 10% sodium citrate. The citrated blood was then centrifuged for 25 minutes at approximately 1500 r.p.m. (625 G). The supernatant fluid was discarded and the cells were washed 3 times with saline solution (0.85% sodium chloride). Final suspensions were made in saline from the packed cells. All guinea pig blood cells were used within 6 hours after collection.

A 1% suspension of blood cells was employed in all tests unless otherwise stated. Such a suspension of guinea pig blood cells was found to contain 175,000 erythrocytes per cmm. Very few white blood cells were present in a 1% suspension of erythrocytes.

In tests for agglutination of cells with the polysaccharides of pneumococci types I and III and influenza virus (PR8), a 0.25% red blood cell suspension was employed.

Leukocyte suspensions.—Guinea pig leukocytes were obtained by inducing exudation into the peritoneal cavities of these animals by injecting intraperitoneally 100 ml of Locke solution containing 0.1% glucose about 15 or 16 hours before sacrificing the animal and harvesting the exudate.[3] The quantity and quality of the exudate was more satisfactory if the guinea pigs were maintained on ascorbic acid (50 mg/day) or were given a heavy cabbage diet for 5 to 7 days before using. The exudate was placed in 50 ml centrifuge

tubes containing 3 to 4 ml of 10% sodium citrate and centrifuged for 10 minutes at 100 r.p.m. (43 G). The cells were washed 3 times with saline. From the packed cells a 1% suspension was prepared for use in the tests. Such suspensions of guinea pig leukocytes were found to contain about 13,000 cells per cmm. The erythrocyte contamination of a 1% suspension of leukocytes was about 600 per cmm. Differential counts made on the guinea pig exudate indicated that 90 to 95% of the cells were polymorphonuclear leukocytes.

Antiserums.—The antiserums were prepared by 10 intraperitoneal injections of 1 ml of the appropriate antigen (a 20% cell suspension) into rabbits (3 injections per week over a period of 3 weeks). The 9 injections were followed by a booster shot after a rest period of one week. An equal amount of sterile glycerine was added to the antiserum as a preservative. It was determined that 50% glycerine in the serum did not give rise to false agglutination. Dilutions of antiserums were made in saline, covering a range of 1:20 to 1:10,240, with a final concentration of 0.5% of antigen cells. The tubes after shaking were incubated at 37.5 C in a water bath for one hour. Readings were again made after the tubes had stood overnight at 5 C.

Agglutinin-adsorption.—The appropriate antiserums were diluted 1:10 and then treated with 1 ml of packed guinea pig erythrocytes or leukocytes per 10 ml of diluted antiserums. After standing overnight at 5 C the supernatant fluid was tested for its ability to agglutinate guinea pig red and white cells. A control was run on the possible adsorption of antibodies by erythrocytes present as contamination in the adsorbing antigen, using 0.02 ml of packed cells in 10 ml of a 1:10 dilution of antileukocyte serum. The results indicated no adsorption of antibody by the contaminating erythrocytes.

Agglutination with influenza virus (PR8).—Influenza virus-infected allantoic fluid from chick embryos was pooled and stored at 5 C. Dilutions of this material were made in saline solution. To 0.5 ml of each dilution of virus material was added 0.5 ml of a 0.25% red blood cell suspension or 0.5 ml of a 1% suspension. Final dilutions of virus covered a range of 1:10 to 1:5120 of infected allantoic fluid. The tubes were shaken immediately and then allowed to stand at room temperature for 90 minutes before reading. The hemagglutination titrations were read by the pattern technique described by Salk.[4] The agglutination of leukocytes was accomplished by mixing virus material and white blood cells and recording the dilution of virus material necessary to cause the clumping of cells.

Elution experiments with influenza virus (PR8).—In all of the elution experiments 1% guinea pig red or white blood cells were used. One ml of PR8 virus was added for every 10 ml of 1% cell suspension used. The flask was allowed to stand at room temperature for 45 minutes and was then transferred to a 37.5 C water bath in order to elute the virus. To insure thorough mixing the 50 ml flask containing the system was shaken every 30 minutes. Aliquot samples were removed at various time intervals. The cells were spun down and the supernatant fluids stored at 5 C for several days to await titration. Dilutions of the supernatants were made in saline. Three sets of dilutions were made for each supernatant. One row of the diluted supernatant was titered with 0.25% chicken erythrocytes, the second, with 0.25% guinea pig erythrocytes, and the third, with 1.0% guinea pig leukocytes.

Agglutination of guinea pig cells treated with influenza virus.—Guinea pig red and white cells were treated with influenza virus (PR8) for 6 hours at 37.5 C, then centrifuged, the supernatant removed and discarded, and the cells washed 3 times with saline. A 1% cell suspension of white blood cells and a 0.25% cell suspension of red blood cells were used for agglutination tests with influenza virus (PR8).

Agglutination of blood cells with pneumococcus type I and III polysaccharides.—Stock polysaccharide solutions containing 10 mg/ml were used in all of the experiments. To 0.5 ml of the varying dilutions of polysaccharide, 0.5 ml of a 0.25% suspension of guinea pig erythrocytes or leukocytes was added. Final dilutions of the polysaccharide covered a range of 1:10 to 1:5120. The tests were read after 90 minutes at room temperature and again after the tubes had stood at 5 C overnight.

Sedimentation of blood cells by polysaccharides.—Citrated guinea pig blood was mixed with various concentrations of S I and S III specific polysaccharides of pneumococci. Final concentrations of S I and S III were 0, 25, 50, 75, and 100 mg %. The mixtures of blood and S I or S III were drawn up in Westergren blood sedimentation tubes of 2 mm bore to a height of 10 mm. The degree of sedimentation was read at the end of one hour as the height of clear plasma. The polysaccharides used were shown to be immunologically reactive in dilutions of 1×10^6 or 1×10^7 by the precipitin test.

Agglutination with navy bean extracts.—Extracts of navy beans (*Phaseolus communis*) were prepared by the method of Li and Osgood.[5] To

4. Salk, J. E. 1944, J. Immunol. **49**: 87–98.

each 0.5 ml of varying dilutions of navy bean extract, 0.5 ml of a 1% suspension of guinea pig erythrocytes or leukocytes was added. Final dilutions of the crude bean extract covered a range of 1:10 to 1:5120. The tubes were immediately shaken and placed in a 37.5 C water bath for one hour. The tubes were read by determining the degree of agglutination after one hour at the above temperature and after they had stood overnight at 5 C.

RESULTS

Agglutination reactions with antiserums.—Direct and cross agglutination tests with red blood cells and leukocytes were done using six different antiserums against each of the two types of cells. The average results of repeated trials given in table 1 clearly indicate, except for low titer antileukocyte serums 3 and 9, that both antigenic differences and similarities exist on the surfaces of the two types of cells.

TABLE 1.—*Direct and cross agglutination of erythrocytes and leukocytes with antiserums.*

	Number	Erythrocytes	Leukocytes
Antierythrocyte serums	1	1:640	1:80
	2	1:1280	1:640
	3	1:1280	1:40
	4	1:1280	1:160
	5	1:640	1:80
	10	1:640	1:80
Antileukocyte serums	1	1:320	1:1280
	2	1:160	1:2560
	3	1:80	1:80
	4	1:160	1:640
	7	1:320	1:640
	9	1:160	1:160
Normal serum			1:20

TABLE 2.—*Adsorption of antiserums by erythrocytes and leukocytes.*

Adsorbed with:	Titered with: RBC	WBC
RBC antiserums + RBC (10%)	−	−
RBC antiserums + WBC (10%)	+	−
Not adsorbed	+	+
WBC antiserums + WBC (10%)	−	−
WBC antiserums + RBC (10%)	−	+
Not adsorbed	+	+

RBC—red blood cells. WBC—white blood cells.

Adsorption tests (table 2) using homologous and heterologous cells supported the results of the agglutination experiments and indicated the presence of both similar and different antigens on erythrocytes and leukocytes.

It should be pointed out that guinea pig exudate leukocytes may vary with respect to their reaction to antiserums. In table 3 results indicate a greater agglutinability of leukocytes obtained from peritoneal exudate stimulated by injection of Locke solution than that of leukocytes accumulated after the injection of saline.

TABLE 3.—*Agglutination of various suspensions of guinea pig leukocytes by antiserums.*

Antiserums	Leukocyte exudate stimulated by: Saline	Locke solution
Antileukocyte	1:320	1:640
	1:320	1:1280
Antierythrocyte	1:160	1:640
	1:80	1:320

*Treatment of cells with trypsin or Calgon.**—According to Chambers[6] the extraneous cell coats of leukocytes are envelopes which may be removed by means of such agents as proteolytic enzymes and possibly calcium-binding solutions. Practical applications of this finding to the field of hematology are beginning to be made.[7]

Experiments were performed in which leukocytes were treated with a proteolytic enzyme, trypsin,† or a calcium-binding solution, sodium metaphosphate (Calgon), in order to remove these

5. Li, J. C. and Osgood, E. E. 1949, Blood, **4**: 670–675.

* Calgon is a product of the Monsanto Chemical Co.

6. Chambers, R. 1940, Cold Spring Harbor Symposium of Quantitative Biology, **8**: 144–153.

7. Morton, J. A. and Pickles, M. M. 1947, Nature, **159**: 779–780.

† Fairchild and Difco Laboratory preparations.

TABLE 4.—*Average agglutination of trypsin- or Calgon-treated guinea pig blood cells with antiserums.*

Antiserums	Antigen	Experiment	Treatment							
			Control		Trypsin		Control		Calgon	
Antileukocyte	Leukocytes	A	1:320	5/4*	1:1280	5/4*	1:480	4/3*	1:320	4/3*
		B	1:320	7/5	1:2460	7/5	1:320	2/2	1:480	2/2
Antierythrocyte	Erythrocytes	A	1:640	11/8	1:1280	11/8	1:640	2/1	1:960	2/1
		B	1:640	9/4	1:1280	9/4	1:480	9/4	1:640	9/4

Experiment series A.—One portion of cells treated with one portion of trypsin or Calgon.
Experiment series B.—Two portions of cells treated with one portion of trypsin or Calgon.
* Number of tests done/number of antiserums used.

extraneous cell coats and thereby possibly alter the agglutination of these cells by their antiserums.

In one set of experiments, 10 or 20% suspensions of cells were treated with three preparations of trypsin having a final concentration of about 1%. The reaction was carried out at 37 C over a period of one hour. The cells were then washed 3 times with saline, diluted to make a 1% suspension and compared in agglutination tests with untreated cells. In a similar manner, 10 or 20% suspensions of cells treated with a 1% solution of Calgon in saline for one hour at 37 C were washed and tested against antiserums.

The results as summarized in table 4 fail to show any significant effect of treating either leukocytes or erythrocytes with Calgon, a strong solvent for calcium salts. On the other hand, repeated tests indicated that both leukocytes and erythrocytes treated with trypsin were more readily agglutinated with homologous antiserums (2 to 4 times). No evidence is available to show the mechanisms involved. The significant point is that both types of cells are similarly affected, although the leukocytes respond to a greater degree to trypsin treatment than do the erythrocytes.

Agglutination of cells with influenza virus.—Considerable progress has been made in defining the probable nature of the chemical material on the erythrocyte to which the influenza virus attaches itself with a resulting hemagglutination. Observations indicating that a polysaccharide complex of the red cell played a major role in this phenomenon have been made by de Burgh et al.[1] Because of the developments in the field of hemagglutination of erythrocytes by viruses, particularly the influenza virus, it seemed reasonable to use this virus as a tool in comparing the surfaces of the erythrocyte and the leukocyte. Several types of experiments have been carried out.

The first experiments were set up to compare the agglutination of erythrocytes and leukocytes of guinea pigs by influenza virus (PR8). As a preliminary experiment on technique it was determined that 0.25 % of guinea pig erythrocyte suspension gave an 8-fold higher titer with a given virus preparation than a 1.5% cell suspension (ti-

TABLE 5.—*Agglutination of leukocytes and erythrocytes by influenza virus (PR8).*

Exp. no.	PR8 virus lot no.	Titer	
		WBC (1%)	RBC (0.25%)
		Guinea pig	
1	A	1:640	
2	B	1:1280	
3	C	1:320	
4	C	1:320	1:2560
5	C		1:2560
6	C	1:320	1:5120
7	D	1:320	
8	D	1:640	1:5120
9	D	1:640	1:2560
10	D		1:5120
11	D	1:1280	1:1280
	Normal allantoic fluid	0	0
		Rat	
1	D	0	
2	D		1:5120
3	D	0	1:2560

ters of 1:2560 and 1:320, respectively). Using 0.25% and 1.5% suspensions of chicken cells, titers of 1:2560 and 1:160 were obtained. These results agree with the findings of Salk.[4]

Using several lots of virus-infected allantoic fluid, agglutination tests were made with various leukocyte preparations and erythrocyte suspensions. The results given in table 5 show that both the leukocytes and erythrocytes of the guinea pig are agglutinated by influenza virus (PR8). About four times as much virus is required to agglutinate the leukocyte as the red blood cell. In two experiments with rat leukocytes, similarly prepared, no agglutination was noted although the red cells of the rat were agglutinated by dilutions of 1:520 and 1:2560 of virus material.

The next step in using influenza virus as a tool in comparing the surfaces of the two types of cells was to determine whether or not the virus could be eluted equally well from both by subjecting the treated cells to a temperature of 37 C. The results of a typical experiment with guinea pig erythrocytes in which the virus was adsorbed at 25 C and then eluted at 37 C are presented in figure 1. The curves give the titers of supernatants of aliquots tested against guinea pig erythrocytes, leukocytes, and chicken red cells. The results of a parallel experiment using

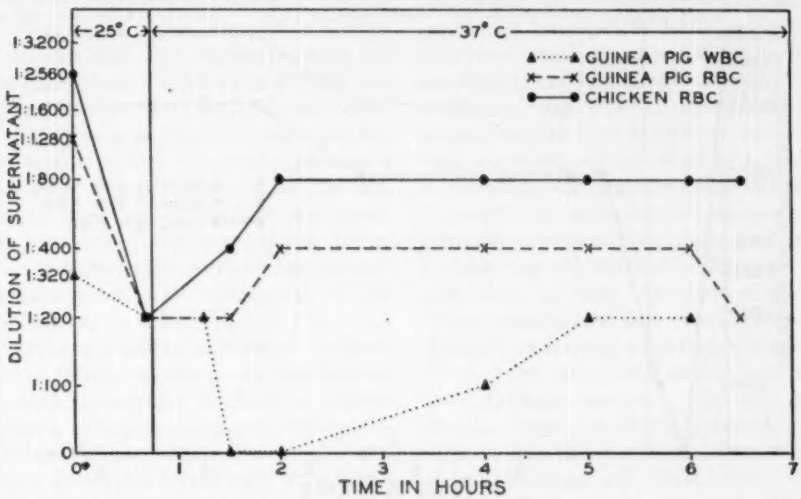

FIG. 1.—Adsorption of influenza virus (PR8) by guinea pig erythrocytes and subsequent elution

guinea pig leukocytes are presented in figure 2. The results of these experiments demonstrate that virus adsorbed by erythrocytes or leukocytes at 25 C is eluted at 37 C more or less completely within an hour. However, it appears that the virus eluted from leukocytes was quite active in agglutinating fresh guinea pig or chicken red cells but not fresh guinea pig leukocytes. This was true to a lesser degree when the virus was adsorbed on erythrocytes.

Hirst[8] found that red blood cells once agglutinated by influenza virus and then freed of it are no longer capable of

8. Hirst, G. K. 1942, J. Exper. Med. **76:** 49–64.

reagglutination by fresh influenza virus. Hirst's explanation for this reaction is:

> The curves of adsorption and elution of influenza agglutinins suggest that the interaction of hemagglutinins and virus occurs in two phases: first, a combination (rapid even at low temperatures) and second, some alteration of the cells accompanied by a separation of the modified cells and the agglutinin. The latter phase appears to be considerably retarded at low temperatures. The modification of the cells in the second phase renders them incapable of combining with more agglutinin, while the released agglutinin is apparently unchanged.

It was of interest then to compare guinea pig erythrocytes and leukocytes as to their capability of being reagglutinated by influenza virus after treatment with and elution of influenza virus. Three experiments were carried out and in all of these it was found that the leukocytes, quite unlike the erythrocytes, were capable of being agglutinated with fresh virus in dilutions of 1:80 to 1:640.

Thus, two differences between the action on and reaction to influenza virus (PR8) of guinea pig erythrocytes and leukocytes have been found. (1) The leukocytes alter the virus, decreasing its ability to reagglutinate other leukocytes but not affecting its action on fresh guinea pig or chicken erythrocytes. (2) Leukocytes can be reagglutinated by fresh virus although the erythrocytes, as Hirst observed, cannot.

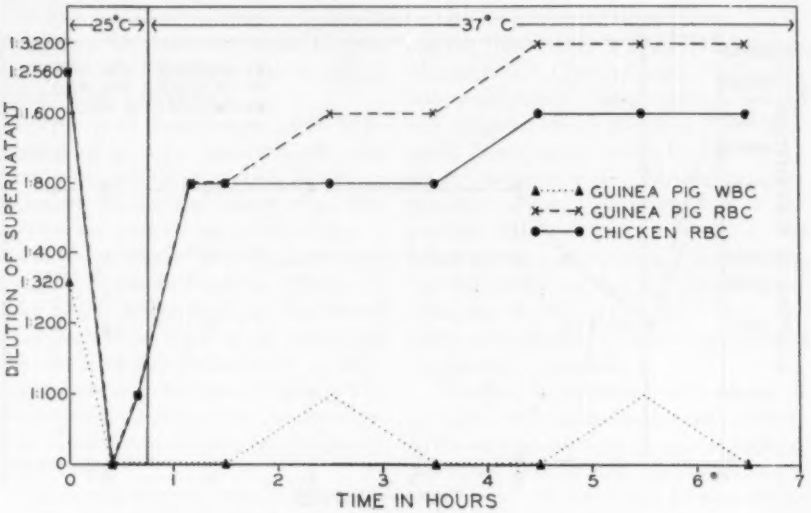

FIG. 2.—Adsorption of influenza virus (PR8) by guinea pig leukocytes and subsequent elution.

Action of pneumococcus polysaccharide on erythrocytes and leukocytes.—Many substances of plant, animal, or microbial origin are known to agglutinate erythrocytes. An increase in sedimentation rate, although a more sensitive test, parallels as a rule the agglutination of red cells. Since pneumococcus polysaccharide has been shown[9] to increase the sedimentation rate of blood cells and since this material is an essential component of virulent pneumococci, its

9. Nungester, W. J. and Klein, L. F. 1937, Proc. Soc. Exper. Biol. & Med. **36**: 315–317.

relative effect on leukocytes and erythrocytes was determined. The results were somewhat surprising. Although a dilution of 1:4000 of polysaccharide type I or III increased the sedimentation rate of red blood cells, a 1:200 concentration was required to agglutinate the red blood cells of guinea pigs. Type I polysaccharide agglutinated guinea pig leukocytes in a dilution of 1:128,000 and type III polysaccharide in a dilution of 1:640. Rat leukocytes were agglutinated by a dilution of 1:4000 of S I and not at all by S III. It may be of interest to note that the guinea pig has at least a million fold greater resistance to type I pneumococci than the rat. Again there are similarities in a qualitative sense, yet quantitative differences between the surface reactions of erythrocytes and leukocytes of the guinea pig do exist.

Phytoagglutinins on blood cells.—Navy bean (*Phaseolus communis*) extract was tested for its relative effects in agglutinating erythrocytes and leukocytes. Using the crude extract it was found that the erythrocytes were from 2 to 4 times as readily agglutinated as were leukocytes from the same animal, the guinea pig. Titers for the extract of 1:2560 and 1:640 were obtained using erythrocytes or leukocytes, respectively.

SUMMARY AND CONCLUSIONS

As a result of studying the effect of various materials, as antiserums, influenza virus, pneumococcus polysaccharide, and navy bean extract in agglutinating guinea pig erythrocytes and leukocytes, certain conclusions are made with respect to possible similarities and differences of surfaces of these two types of cells.

It appears that there are similar components on the surfaces of erythrocytes and leukocytes of the guinea pig. This conclusion is supported by cross agglutination with antiserums and similarity of effect in producing agglutination by influenza virus (PR8), pneumococcus polysaccharide, and navy bean extract. Also the similarity of effect of trypsin in increasing the agglutinability of both types of cells by antiserums suggests a common component on their surfaces. Considering the marked differences in physiology of these two types of cells, it is interesting that they should react so similarly to the biological reagents used.

However, certain differences between the cells were observed. Antibody adsorption tests clearly established differences in components on the surfaces of erythrocytes and leukocytes. The greater effect of pneumococcus polysaccharide in agglutinating leukocytes than erythrocytes was another indication of a surface difference. Finally, leukocytes alone altered influenza virus so as to decrease, quantitatively at least, its ability to agglutinate leukocytes without appreciably altering its effect on erythrocytes.

THE PARASITEMIA IN EXPERIMENTAL TOXOPLASMOSIS

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The discovery by Wolf et al[1] in 1939 that *Toxoplasma gondii* is the etiological agent of a highly fatal disease of infants has led to the description of an increasingly large number of cases in recent years. While the majority of cases have been neonatal, characterized by hydrocephalus, cerebral calcifications, and chorioretinitis, a few adult cases have also been reported. The majority of cases of adult infection appear, however, to go unnoticed; and mothers who show no evidence of disease can transmit the infection through the placenta to their offspring. While case studies are clarifying the clinical picture of toxoplasmosis, and while a number of studies of the immunologic diagnosis of the infection have been reported, little is known about the biology of the parasite or its method of spread beyond the fact that it occurs in a wide variety of mammals and birds. Especially because the disease is so acute in infants and does so much damage before diagnosis is possible, the preventive aspect of the problem is extremely important and knowledge of this sort is very necessary. With this end in view, studies on the biology of *T. gondii* have been initiated at this laboratory. One aspect of the problem has been the parasitemia in *Toxoplasma* infections.

The presence of *T. gondii* in the peripheral blood of infected animals has been recorded by a number of investigators. Microscopic demonstration of the parasites in the blood of infected birds was reported by Carini,[2] Nicolle and Conor,[3] Arantes,[4] and Wolfson.[5] The organisms were found free in the plasma and/or in red blood cells or monocytes. Reis and Nobrega[6] found pigeon blood infective as early as two days after intramuscular inoculation. Levaditi and Schoen[7] also reported finding toxoplasmas in the blood plasma of chick embryos. In the case of mammalian infections, parasites have been demonstrated microscopically only rarely in the blood (in the gondi, Nicolle and Conor[3]), but subinoculations of blood into receptor animals have been used to show their presence in this situation. Bland,[8] for instance, reported transmitting toxoplasmosis from rabbit to rabbit by blood inoculation and stated that he never saw toxoplasmas in the blood. Sabin and Olitzky[9] were able

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1. Wolf, A., Cowen, D. and Paige, B. 1939, Human toxoplasmosis: occurrence in infants as an encephalomyelitis. Verification by transmission to animals. Science, **89** (2306): 226–227.
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8. Bland, J. O. W. 1931, Glandular fever II. The protozoal nature of the experimental disease. Brit. J. Exper. Path. **12**: 311–319.
9. Sabin, A. B. and Olitsky, P. K. 1937, *Toxoplasma* and obligate intracellular parasitism. Science, **85** (2205): 336–338.

by mouse inoculation to show the presence of toxoplasmas in the blood of rodents and monkeys which had been inoculated by various peripheral routes, but not following intracranial inoculation. Wolfson[8] found the blood of rabbits infective for duck embryos. Pinkerton and Henderson[10] produced an infection in guinea pigs by the inoculation of blood from a patient dying of toxoplasmosis.

On the other hand, Pixell[11] was unable to transfer *T. gondii* with the peripheral blood of infected animals but was successful with heart's blood. Cross and Anigstein[12] state that they also did not find parasites in the bloodstream after careful scrutiny. These latter authors emphasize the intracellular localization of *Toxoplasma* and speculate that the transfer of parasites by means of whole blood might be due to the presence of infected leukocytes or to contamination of the blood by infected "sheath" cells which lie around the vessel walls. They regard migratory cells of the reticulo-endothelial system as the normal mode of migration of toxoplasmas within the body of the host.

Our interest in this problem developed from consideration of the possibility of transmission of toxoplasmosis by bloodsucking arthropods. No quantitative information was available on the number of parasites, if any, which might be found in the blood of toxoplasmic animals at particular times in the course of the disease. It was essential to accumulate such data for two reasons. First, knowledge as to the time of highest parasitemia was necessary for the exposure of donor animals to arthropods during the most propitious period to ensure opportunity for such arthropods to acquire parasites with their blood meal. Secondly, differences in the parasitemia among laboratory animals might be ascertained which would lead not only to the selection of the most appropriate host for such studies but also to possible clues as to the most likely vectors. In addition to these considerations, knowledge of the manner of spread of toxoplasmas within the body might be of importance from the standpoint of chemotherapy and immunology.

In the study reported here, estimations have been made of the number of parasites in the blood of infected mice, rabbits, rats, chicks, and pigeons on successive days after inoculation. The parasites were demonstrated in the blood by the technique of animal inoculation.

MATERIALS AND METHODS

The RH strain of *T. gondii* was used in most experiments. This was received in February, 1947 through the offices of Dr. Albert Sabin and Dr. Isaac Ruchman, of the Children's Hospital Research Foundation, Cincinnati, Ohio. In two experiments with mice, a strain of *Toxoplasma* isolated from a dog by Dr. T. L. Perrin, then of the Pathology Laboratory, National Institutes of Health, in February, 1948, and maintained by us thereafter, was used; this has been designated as the "D" strain.

In some early experiments, inoculums for infecting animals were prepared merely by grinding infected tissues in Locke's solution. No quantitative estimate was made of the number of organisms given. In most instances, the inoculums were prepared from the peritoneal exudate of intraperitoneally infected mice. Counts of the toxoplasmas in this material were made in a Neubauer-Levy counting chamber. Usually a 1:100 dilution of the peritoneal exudate was satisfactory for the counts. All organisms in 25 large squares in the chamber were counted, and the count was repeated 3 times. In order to avoid variation in counts, an attempt was made to use

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TABLE 1.—*The parasitemia in mice infected with* T. gondii.

Exp. no.	Mouse no.	Inoculated		Highest blood dilution causing infection in receptor mice on day*																			
		Date	Size of inoculation	1	2	3	4	5	6	7	8	9	10	11	12	13	14	77	78	79	80	83	84	85	86
A. Acutely infected mice																									
1	1	1/20	0.2 ml brain suspension	10†			D‡																		
	2	1/20	0.2 ml brain suspension		1000†		D																		
	3	1/20	0.2 ml brain suspension			100	D																		
2	4	2/2	0.2 ml brain, liver, spleen suspension	10				D																	
	5	2/2	0.2 ml brain, liver, spleen suspension		100			D																	
	6	2/2	0.2 ml brain, liver, spleen suspension			1000		D																	
	7	2/2	0.2 ml brain, liver, spleen suspension				1000	D																	
3	8	4/8	0.2 ml peritoneal fluid (1:1)§						1000		D														
4	9	10/16	1500 parasites	N¶	N	N	100	1000	D																
	10	10/16	500 parasites	N	N	10	10	100	1000	D															
	11	10/16	100 parasites	N	<10‖	N	N	1000	1000	1000	1000	D													
B. Mice with asymptomatic infections																									
5	12	4/16	0.2 ml brain, liver, spleen suspension**										10				100								
	13	4/16	0.2 ml brain, liver, spleen suspension**											100											
	14	4/16	0.2 ml brain, liver, spleen suspension**												10										
6	15	4/20	20 parasites															N	N	N	<10‖	N	N	N	10

* Day of infection = day 0.
† Heart's blood tested; all others were tail blood.
‡ D = died.
§ "D" strain *T. gondii*.
‖ 1:10 dilution negative, 1:100 dilution positive; hence parasites were really below 1:10 level.
¶ N = negative.
** "D" strain *T. gondii*, refrigerated overnight before inoculation.

only exudates in which the toxoplasmas were present mostly as individuals rather than in clumps within white cells or their remains. Usually 3- or 4-day-infected mice showed such material with predominantly free parasites. While the fact that some clumping did occur precluded great accuracy, variation generally did not exceed 10%. After the number of toxoplasmas in the peritoneal fluid was ascertained, it was merely a matter of preparing the proper dilutions of the suspension to obtain the desired inoculum. All dilutions were made in Locke's solution. The time occupied in counting the parasites and making the dilutions was about one hour, and the animals were inoculated immediately after these preparatory steps were completed so that death of the toxoplasmas from standing in saline at room temperature could be minimized. (In studies to be published it has been found by the authors that small numbers of toxoplasmas lose their infectivity for mice after standing in saline for several hours.)

The most extensive experiments were performed with rabbits because these animals were already in use as hosts in studies on the transmission of toxoplasmosis by arthropods. These animals were New Zealand Whites, raised at the National Institutes of Health, and weighing generally between 4 and 6 pounds. They were infected by a variety of routes: intraperitoneal, intracutaneous, subcutaneous, intramuscular, intravenous, and intracerebral. The other animals were infected by the intraperitoneal route. White rats weighing about 150 g were used. The mice were 4-week-old females weighing between 15 and 19 g. The pigeons were White Carneaux, 6 to 9 weeks old at the time of inoculation. The chicks were New Hampshire Reds, 6 to 14 days old, weighing 13 to 17 g. The size of the inoculums given these animals in the various tests will be indicated below. In all cases except mice, 0.5 ml of the appropriate dilution of peritoneal exudate was the vehicle.

Determinations of the parasitemia in the infected animals were made by the intraperitoneal inoculation of dilutions up to 1:1000 of whole blood into receptor mice. In the case of rats, peripheral blood was obtained from the tip of the tail by snipping off the end and allowing the blood to flow into a small graduated tube containing 0.5 ml of 2% sodium citrate in Locke's solution until the 1 ml mark was reached. This then constituted a 1:2 dilution of the blood. In the case of mice, except for one experiment in which heart's blood was used, 0.1 ml or 0.2 ml of tail blood was obtained by a technique similar to that employed for rats or by drawing the blood into the barrel of a syringe containing the sodium citrate solution. Rabbits were bled from the ear vein, and the chicks and pigeons from the jugular or wing veins. In these cases, 0.2 ml of the sodium citrate solution was first drawn into a 2 ml syringe; then 0.2 ml of blood was drawn, and the syringe immediately filled with Locke's solution to the 2 ml mark. The suspension was homogenized by allowing a few air bubbles to move back and forth in the barrel, and this was then a 1:10 dilution of the whole blood. Further dilutions were made from it.

To test the infectivity of each blood dilution, 0.5 ml was inoculated intraperitoneally into each of two mice. Each such pair of mice was then placed in a separate battery jar and supplied with food and water. In the earlier experiments, after one week one of these mice was killed and impression smears were made of its brain, liver, and spleen. The smears were fixed in methyl alcohol and stained with Giemsa. If any peritoneal exudate was found, this was examined immediately in the fresh state under the 4 mm objective of the compound microscope. After one or two more weeks, the second mouse was killed and examined in the same manner, and a combined suspension of the brain, liver, and spleen was subinoculated into two more mice. In later experiments, a second pair of subinoculated mice received material from both of the first pair. These animals were then killed and examined at one and two weeks. All slides were studied for at least 10 minutes before being ruled negative. While this method of searching for parasites does not exclude the possibility of missing positive cases, it was not feasible to carry the examinations further.

Details of a few corollary experiments on the presence of toxoplasmas in the subcutaneous tissues of animals exhibiting no parasitemia will be presented below.

EXPERIMENTAL RESULTS

The parasitemia in mice

The results of studies on the number of parasites in the blood of *Toxoplasma*-infected mice are summarized in table 1. In these experiments with mice, the determinations of parasitemia were in some cases made on the same animal which was bled on successive days (mice nos. 9, 10, 11, and 15). In the other experiments the estimations of parasitemia were made from a series of animals inoculated at the same time with equal doses of the same infective

material. A different mouse was then bled each day thereafter. In all except the first two mice of experiment no. 1, peripheral blood obtained from the tip of the tail was used for the tests.

The data in table 1 are grouped on the basis of survival or death of the infected mice. Among those in group A, all of which succumbed to toxoplasmosis, a difference is evident in the survival time and in the time of appearance of parasitemia, dependent on the size of the inoculum. Following infection with heavy inoculums, the blood was infective on the first day thereafter, and death occurred in 4 or 5 days. When lighter inoculums were used, the parasitemia developed more slowly and, correspondingly, the mice survived a few days longer. In almost all cases the blood was infective 3 or 4 days before the death of the mouse, and generally there was a progressive increase in parasitemia to a level at which 0.5 ml of a 1:1000 dilution of the blood produced infections in receptor animals.

Among the mice in group B with asymptomatic infections, the 3 animals in experiment 5 were survivors of a group of 5 which received inoculums of "D" strain *T. gondii* in a suspension of infected mouse tissue which had been refrigerated overnight. Refrigeration probably resulted in reduction in the number of viable parasites in the tissue. One mouse in experiment 6 was the sole survivor of a group of 4 mice which had each received 20 toxoplasmas of the RH strain. The parasitemia in these mice did not reach the high level found in animals with acute infections. Nevertheless, dilutions of 1:10 or 1:100 of the blood of the animals in experiment 5 were found infective on 4 days during the second week after inoculation. These animals were found still to harbor parasites in the liver, spleen, and brain when they were sacrificed in the fourth week after inoculation. On the other hand, the blood of mouse no. 15 was found infective in low dilution on 2 of 8 tests during the third month after inoculation, and its tissues were found negative when it was sacrificed on the 104th day. Although the data on asymptomatic infections in mice are meager, it appears that such mice show a moderate parasitemia in the early period following inoculation, after which parasites are found in the blood only rarely and in small numbers.

Rats inoculated with *T. gondii* manifest only a slight transient illness characterized by a roughened coat and apparent depression lasting for two or three days during the second week after infection. A few studies on the parasite blood level in rats were carried out. In all cases the blood was drawn from the tail as described above, but because of the low parasitemia expected in these animals, low dilutions of 1:2 or 1:3 of whole blood were tested for their infectivity for mice. The results on 3 rats

TABLE 2.—*The parasitemia in rats infected with* T. gondii.

Animal no.	Inoculated		Highest dilution of blood producing infection in receptor mice on day*																				
	Date	Number of parasites	5	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	34
R-2	4/13	5000	2	2	N†	N	N	N	N	N	N	N	N			N	N						
R-1	1/4	1500							2					N	N								N
R-3	3/18	5000														2	N	N	N	N	N	3	

* Day of infection = day 0.
† N = negative.

are shown in table 2. It can be seen from these data that the parasitemia in rats with chronic toxoplasmosis is very low and sporadic. In all but one case, studies performed on the sacrificed animals showed the presence of toxoplasmas in the spleen, liver, and brain, which are the organs routinely studied in our investigations. Some rats have been found to harbor toxoplasmas as long as seven months after infection.

number of experiments were performed in which large and small inoculums were used to infect the rabbits by various routes. Large inoculums consisted of suspensions of infected tissue or of 100,000 toxoplasmas. Small inoculums ranged from 400 to 3000 organisms. The results of these experiments are presented in table 3. The data are grouped according to the route of inoculation.

Rabbits infected with heavy inocu-

TABLE 3.—*The parasitemia in rabbits infected with* T. gondii.

Rabbit no.	Inoculated		Highest blood dilution causing infection in receptor mice on day*											
	Route†	Number of parasites	1	2	3	4	5	6	7	8	9	10	11	14
20	IP	2 ml. brain, liver, spleen suspension	N‡	100	100	100	100		D§					
284	IP	100,000	N	10	10	100	1000	D						
288	IP	100,000	N	10	100	1000	1000	D						
285	ID	100,000	N	N	10	100	1000	D						
286	SC	100,000	N	N	100	100	100	D						
287	IM	100,000	N	N	1000‖	1000	1000‖	D						
289	IV	100,000	10	100	1000	1000	D							
221	IC	1200	N	N	N	100	10	1000	D					
280	IP	1500			100	N	100	1000	D					
281	IP	1500			N	100	N	100	D					
199	IP	1500	N	N	N	100	100	100	100	1000	1000	D		
178	IV	3000	N	100	10	100	1000	1000	1000	1000	D			
185	IV	500	N	N	10	<10	N	1000	1000	1000	D			
229	IM	1500	N	N	N	N	1000	100	1000	100	1000	D		
123	ID	400	N	N	100	10			1000	1000	D			
261	ID	1500			N	100	100	100	1000		D			
71	ID	500	N	N	N	N			N	N	10	100	10	K¶

* Day of infection = day 0.
† IP = intraperitoneal. ID = intradermal. SC = subcutaneous. IM = intramuscular. IV = intravenous. IC = intracranial.
‡ N = negative.
§ D = died.
‖ 1:1000 dilution positive, 1:100 dilution negative; hence parasites were really below the 1:100 level.
¶ K = killed; found negative on subinoculation into mice.

In one experiment designed to test for the presence of toxoplasmas in the skin of inoculated rats, a series of 6 rats was inoculated subcutaneously with 5000 toxoplasmas on the right side of the back. Then, one of these animals was killed each day from the 3rd through the 7th, and on the 10th day, and the subcutaneous tissues of the left side of the back were inoculated into receptor mice. No infections in the mice were produced in this manner.

The parasitemia in rabbits

The most extensive studies on the parasitemia in mammals were carried out on rabbits. The reason for this choice has already been stated above. A

lums survived for 5 to 7 days,—in the great majority of cases for 6 days. In all instances the blood of these animals was found infective on the third day and occasionally on the second day. There was a progressive increase in parasitemia usually to the 1:1000 level which was reached on the day before death, although in 2 cases the higher dilutions of blood were not uniformly infective for mice.

In general, rabbits inoculated with small numbers of parasites survived a few days longer than those which received heavy inoculums. It may be mentioned here that the survival time of a large number of other rabbits inoculated by various peripheral routes

TABLE 4.—*The parasitemia in pigeons infected with* T. gondii.*

Bird no.	Highest dilution of blood producing infection in receptor mice on day†																					
	1	2	3	4	5	6	7	8	9	10	13	14	15	16	17	19	20	22	24	28	29	44
54	N‡	N	<10§			N	100	1000	1000	1000	1000	1000	1000		1000		1000		1000	N		
31	N	N	10‖	100§	100	100	100	100		1000	1000		N		N		N		N		N	100
90							10‖				1000							10‖				
94	N	N			1000	100	100	1000	1000		1000	1000	1000	1000		1000	D					
93							1000					D¶										
96	N	N	N	10	10	100	100	1000		D												

* All pigeons were infected with 5000 *T. gondii* intraperitoneally.
† Day of infection = day 0.
‡ N = negative.
§ One receptor mouse inoculated with 1:1000 dilution positive; others all negative; therefore parasitemia was below 1:10.
‖ Highest dilution tested.
¶ Died.

TABLE 5.—*The parasitemia in chicks infected with* T. gondii.

Bird no.	Inoculated		Highest dilution of blood producing infection in receptor mice on day*																				
	Date	Number of parasites	1	2	3	4	5	6	7	9	10	11	12	13	14	17	18	19	20	21	24	25	28	29
1	6/13	1500	10		N†	10			D‡															
2	6/17	1500				N	10	N	10		N	N	N	10	10		N	N	N	N	N	N	N	
3	6/17	1500				N	10	10	100		100	N	N	10	N		10	N	N	N	N	N	N	
C101	8/5	3000			N	N	N	N	10		N	N	N	N	N	10§	N	N	N	N	N			
C102	8/5	3000			N	N	N	N	100		N	100	N	N	N	1000	N	N	N	N	N			
TH443	10/3	3000	N	N	10§	N		1000	1000	D														
TH444	10/3	3000	N	10§	N	100		1000	1000	D														

* Day of infection = day 0.
† N = negative.
‡ D = died.
§ Highest dilution tested.

with similar numbers of parasites and used for other purposes has been found to fall in the same range of 7 to 10 days, and no correlation has been found between the route of inoculation and the survival time. It can be seen from table 3 that there was no constant difference in the time of appearance of parasites in the blood which could be attributed to differences in the route of inoculation.

While some exceptions may be noted, the blood of the majority of these rabbits was found infective on and after the fourth day after infection. A progressive increase in parasitemia to the same high level of 1:1000 was found in all cases. In most cases, also, this high parasite blood level was found two or three days, and sometimes even four days, before death.

Rabbit 71 was the only rabbit in this series which survived an infection. This animal and another rabbit which was infected at the same time with the same inoculum have been the only survivors in a series of over 450 rabbits used in our investigations. It is believed that survival in these cases was due to a reduction in the number of viable organisms in the inoculum, due to centrifugation and handling, rather than to immunity. It appears from the data on this rabbit that some parasites do appear occasionally in the blood in latent infections in these animals, but that, as in the case of mice, their number is lower than in acute infections.

In all of the experiments listed in table 3, the 1:1000 dilution of blood was the highest tested for infectivity. This was done because the time of survival of mice inoculated with this dilution was generally longer than would be anticipated were they inoculated with even as few as 10 toxoplasmas. Also, the demonstration of infection in such mice was sometimes made only by subinoculation. Therefore, it was expected that dilutions of blood higher than 1:1000 would not be found to be regularly infective. In order to check this point, however, 2 rabbits were inoculated with 100,000 toxoplasmas and 4 rabbits with 1500 parasites; then studies on the infectivity of 1:2000, 1:5000, and 1:10,000 dilutions of the blood of these animals were made on the last few days before their anticipated death. In 5 cases, on the day before death the 1:2000 dilution was found infective; in 3 cases, the 1:5000 dilution was found infective. The 1:10,000 dilution failed in every instance to produce an infection in the receptor mice. It has therefore been demonstrated that the 1:1000 level selected as the upper limit in the earlier tests represents a fair approximation of the highest level of parasitemia in rabbit toxoplasmosis.

Since some controversy exists in the literature as to whether or not toxoplasmas migrate through the body as free organisms in the blood stream, an attempt was made to find parasites in blood smears prepared at a late stage in the course of the disease. Smears were made from rabbit 178 on the 8th day of infection, and after a high parasitemia was demonstrated by the subinoculation technique, these slides were searched for parasites. On each of two slides which were examined over a period of several hours, one free toxoplasma was found. No infected white blood cells were seen.

The parasitemia in birds

The results of studies of the parasitemia in pigeons and chicks are summarized in tables 4 and 5. In all cases the birds were inoculated by the intraperitoneal route. Pigeons were infected with 5000 toxoplasmas, while the infective dose for chicks was either 1500 or 3000 parasites.

Of the 6 pigeons listed in table 4, the

first 3, nos. 54, 31, and 90, manifested no symptoms of disease and survived for over 3 months until sacrificed. The other 3 birds died of acute toxoplasmosis which was characterized by an abrupt onset only a few days before death. The time of survival of these pigeons varied from 10 to 20 days.

The striking thing to be noted from the data in table 4 is that regardless of survival or death of the infected pigeons, all the birds showed a high parasitemia. In the pigeons which succumbed to *Toxoplasma* infections, a parasitemia developed 4 or 5 days after infection and reached a high level at least a few days before death. In the birds which survived, an equally high level of parasitemia appeared at the end of the first week of infection, persisted for one or two weeks thereafter, and then subsided abruptly. In all cases, tests made during the second week after infection showed a high parasite blood level.

The positive finding on the blood of pigeon no. 31 on the 44th day after infection indicates that in pigeons with latent toxoplasmosis the blood may occasionally be infective after the earlier abrupt drop in parasitemia. Parasites may remain in the internal organs for at least 3 months after infection, when the blood is no longer infective. Pigeons 54 and 90 were sacrificed 3 and 3½ months, respectively, after infection. Both were found to harbor toxoplasmas in the brain, liver, and spleen. Scrapings from the skin of no. 90 were also made. These did not produce infections in receptor mice.

The data in table 5 show that parasitemia develops rapidly in chicks with acute toxoplasmosis. In the three birds which succumbed to the infection, nos. 1, TH 444, and TH 443, the blood was found infective on the first, second, and third days respectively. A high level of parasitemia was also found in the two chicks which were tested two and three days before death.

In those cases in which the chicks survived infection with *T. gondii* (nos. 2, 3, C101, and C102) the parasitemia was lower and less constant than in pigeons with asymptomatic infections. There was no period, such as that found in the case of pigeons, during which the blood of chicks was regularly found infective. In general, no parasitemia was found in any of these birds earlier than the 5th nor later than the 18th day after infection.

Two months after infection, chicks nos. 2 and 3 were sacrificed and their tissues examined for the presence of toxoplasmas by direct smear and by subinoculation into mice. Toxoplasmas were found in the liver and spleen of chick no. 2 and in the spleen of chick no. 3. They could not be demonstrated in the brains of these birds. Studies were also made of the skin. An area of approximately 3 square inches was removed from the backs of the chicks and was scraped with a knife to remove the adherent tissues. These were then macerated in a mortar in Locke's solution and inoculated into mice. No infections were produced in this manner.

While some pigeons and chicks did succumb to *Toxoplasma* infections, it appears more interesting that others had apparently asymptomatic infections but were nevertheless demonstrated to have a high parasitemia. The possible significance of these findings will be discussed below.

DISCUSSION

The data reported here add some support to the idea that, among the various conceivable methods of spread of *T. gondii*, transmission by blood-sucking vectors merits investigation. It is obvious from these results that in any such study careful attention should

be given to the parasitemia in the donor hosts. The choice of animal and the time at which arthropods are allowed to feed are of considerable importance, especially in the case of arthropods which take their blood meal rapidly and infrequently. While some variation has been encountered in the work reported here, it is believed that these data will contribute significantly to the effective prosecution of investigations with arthropods and may also indicate some useful leads in the selection of vectors for testing.

In regard to the choice of animal, it is evident that although *T. gondii* has been found to occur frequently in rats (Perrin et al[13]), these animals would be practically useless in a study of transmission by bloodsucking vectors. The same would be true of mice or rabbits with latent toxoplasmosis. If mammals are to be used in such an investigation, mice or rabbits with acute toxoplasmosis should serve the purpose. If the choice need not be limited to mammals, pigeons should be preferred to chicks.

As to the time during the course of the infection at which the arthropods should be allowed to feed, certain suggestions may be made on the basis of current data. In the case of mice which receive inoculums of large numbers of parasites, the blood may be expected to be infective in a titer of 1:100 or higher as early as the third day of infection. If the mice are infected with fewer parasites, the most propitious time to allow arthropods to feed would be from the fourth or fifth day on. In the case of rabbits, the opportunity for arthropods to acquire toxoplasmas in feeding on these animals would be best after the fourth day. The somewhat longer survival time of mice and rabbits infected with small numbers of toxoplasmas makes these animals suitable for use with slow-feeding arthropods. Ticks, for example, require many days to engorge completely, and the use of rabbits infected with small inoculums provides better opportunity for them to complete their feeding.

In regard to birds, pigeons should serve satisfactorily as donor hosts during the second week of infection. Although some birds were found positive earlier and later, during this period in all of our experiments the blood of pigeons has been found infective in high dilution. Because of the small number of birds studied up to now it cannot be stated that the parasitemia in pigeons has been adequately measured. The birds should be tested from time to time in order for the investigator to have assurance that a parasitemia is present at the appropriate period in the experiment.

It cannot be presumed that parasites may be picked up from the subcutaneous tissues, since our findings on skin scrapings of parasitized birds and mammals have been consistently negative in the absence of parasitemia.

All of the rodents examined in this study manifest a high blood parasite level only when they are acutely ill from toxoplasmosis. Rodents with asymptomatic infections show a very low and sporadic parasitemia. The striking characteristic of *Toxoplasma* infection in pigeons, on the other hand, is the occurrence of a high level of parasitemia persisting for up to two weeks in birds which show no symptoms of disease. This information suggests that birds may serve efficiently as reservoirs of *Toxoplasma* and that arthropods which attack birds as well as mammals should be investigated as possible vectors.

Concerning the numbers of parasites

13. Perrin, T. L., Brigham, G. D. and Pickens, E. G. 1943, Toxoplasmosis in wild rats. J. Infect. Dis. **72**: 91–96.

in the blood, as interpreted from the dilutions found infective for mice, a positive result with 0.5 ml of a 1:1000 dilution would indicate the presence of at least 2 parasites per cmm of whole blood. This figure is based on the assumption that the inoculation of one parasite is sufficient to produce an infection. Although information is not available on the minimum number of toxoplasmas required to infect mice regularly, it is believed that 5 would be a fair figure for inoculations by the intraperitoneal route. Therefore, the estimate of the number of toxoplasmas per cmm of blood when the 1:1000 dilution is positive may be increased to 10. Since dilutions of 1:2000 and 1:5000 of the blood of infected rabbits were found infective on some occasions, the parasitemia may reach a level of 50 toxoplasmas per cmm. Further work on pigeons in regard to this point is indicated.

In addition to the importance of the parasitemia in infected animals as related to the possible transmission of toxoplasmosis by bloodsucking vectors, the results presented here have some bearing on a few other points of interest. For one thing, it has been demonstrated by the studies on rabbits that a parasitemia develops in these animals to the same degree regardless of the route of inoculation, whether it be peripheral or intracranial. While it has been shown by some workers[7] that migration of toxoplasmas may take place along nerve trunks, the parasites produce a generalized infection even when introduced into the central nervous system.

Also, these results do not lend support to the idea that toxoplasmas are transported mainly by migratory cells of the reticulo-endothelial system along nerve channels and extravascularly along the larger blood vessels.[12] While it is recognized that blood specimens may become contaminated with cells from the periphery of the vessels, it is believed that the consistent findings reported here on the infectivity of various dilutions of blood would not have been obtained were the toxoplasmas present only extravascularly. These results are more easily explained on the basis of the presence of parasites within the blood stream, where they might occur free or in leucocytes. In our study of blood smears from animals exhibiting parasitemia, the organisms were found free in the plasma. It has also been shown[14] that *T. gondii* has independent motility. It is therefore entirely likely that toxoplasmas are capable independently of entering the circulation.

SUMMARY AND CONCLUSIONS

Toxoplasma gondii produces a generalized infection regardless of the route of inoculation. The parasites can be found free in the blood plasma, and it is believed that they are capable of independent migration within the host.

In mice and rabbits suffering acute toxoplasmosis, parasites are found in the blood 3 or 4 days before death, and there is a progressive increase in parasitemia to a level at which 0.5 ml of a 1:1000 dilution of the blood produces infections in receptor animals. The highest dilution of rabbit blood found infective was 1:5000.

Mice with asymptomatic *Toxoplasma* infections exhibit lower blood parasite levels in the early period following inoculation, after which parasites are found in the blood only rarely and in small numbers. A low blood parasite level was also found in one rabbit which survived infection.

14. Guimarães, F. N. and Meyer, H. 1942, Cultivo de "Toxoplasma" Nicolle and Manceaux, 1909, em culturas de tecidos. Rev. Brasil. de biol. **2** (1): 123–129.

While rats are susceptible to infection with *T. gondii*, they manifest only mild and transient symptoms and show a very low and sporadic parasitemia. Toxoplasmas have been isolated from rats as late as 7 months after infection.

All of 6 pigeons tested, regardless of survival or death, showed a high parasitemia. The blood of all the birds tested was positive in high dilutions during the second week after infection, while some were found positive earlier and later as well. In surviving pigeons, there was an abrupt disappearance of parasites from the blood after the third week, but toxoplasmas were found in the brain, liver, and spleen 3½ months after infection.

Chicks with acute toxoplasmosis showed blood parasite levels similar to those in pigeons, while those with asymptomatic infections were found to have parasites in the blood at somewhat lower levels and more irregularly. Parasites remain in the liver and spleen of surviving chicks for at least two months after infection.

The subcutaneous tissues of infected rats, pigeons, and chicks were found not to harbor parasites in the absence of parasitemia.

These data lend support to the theory that *T. gondii* may be transmitted by bloodsucking vectors. In the choice of animals as donor hosts for arthropods in tests of this theory, the rat would be of little use. Acutely infected mice or rabbits should serve satisfactorily during the latter part of their infections. Pigeons should be satisfactory donors during the second week after infection.

The high parasitemia found in pigeons with asymptomatic infections suggests that birds may serve efficiently as reservoirs of *Toxoplasma* and that arthropods which feed on both avian and mammalian hosts should be studied as vectors.

EFFECT OF HIGH CONCENTRATIONS OF NICOTINIC ACID AND NICOTINAMIDE ON GROWTH OF SOME STREPTOCOCCI AND PNEUMOCOCCI

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In a previous publication[1] it was reported that in a simple chemically defined medium, growth of a number of the gram-negative enteric bacteria, together with a few other miscellaneous types, was markedly retarded or completely inhibited by 5000 or 10,000 μg per ml of nicotinic acid or nicotinamide. The medium was composed of ammonium phosphate, glucose, salts, and in some cases small amounts of one or two additional vitamins or amino acids where called for by the specific needs of the test microorganism.

This environment represents an extreme example of nutritional imbalance, for the cells must carry on metabolism and synthesize other vitamins and cellular materials in the presence of a great excess of one vitamin. It was found also that the inhibition caused by excessive amounts of vitamin was nullified by the addition of 0.1% yeast extract, that is, in the presence of 10,000 μg of the vitamin types such as the dysentery bacillus and others, which ordinarily have a relatively simple vitamin requirement, were more exacting and required yeast extract for growth. The yeast extract could not be completely replaced by a mixture of known vitamins and related substances. Such results serve to emphasize the effect of environment on the vitamin and other nutritive requirements of microorganisms.

In the present study the work was extended to include tests in a "rich" medium which supplied many nutritive substances preformed. The culture medium was a veal infusion broth with 1.0% Bacto peptone and 0.5% glucose. The organisms used in the current tests were streptococci and pneumococci which grew quite readily in this medium.

To the veal infusion medium nicotinic acid or nicotinamide was added in varying amounts up to 20,000 μg per ml. The medium was adjusted after addition of the vitamin to pH 7.3 to 7.5 for many of the tests, though in some cases other pH levels were used as specified later. The inoculum consisted usually of 0.001 ml of a 24-hour culture in the veal infusion medium. Incubation was at 37 C. Growth was measured by turbidity using a Lumetron colorimeter and a 530 mμ filter. Readings were made daily for the first 3 or 4 days of incubation and then again at the 6th or 7th day.

Results with some representative cultures are shown in figures 1 to 4. Growth of a group A hemolytic streptococcus (fig. 1) was moderately retarded by 10,000, 15,000 or 20,000 μg of nicotinic acid per ml. Nicotinamide, on the other hand, exerted definite inhibition in like amounts. Tests with another strain of hemolytic streptococcus, not shown in the figure, yielded similar results except

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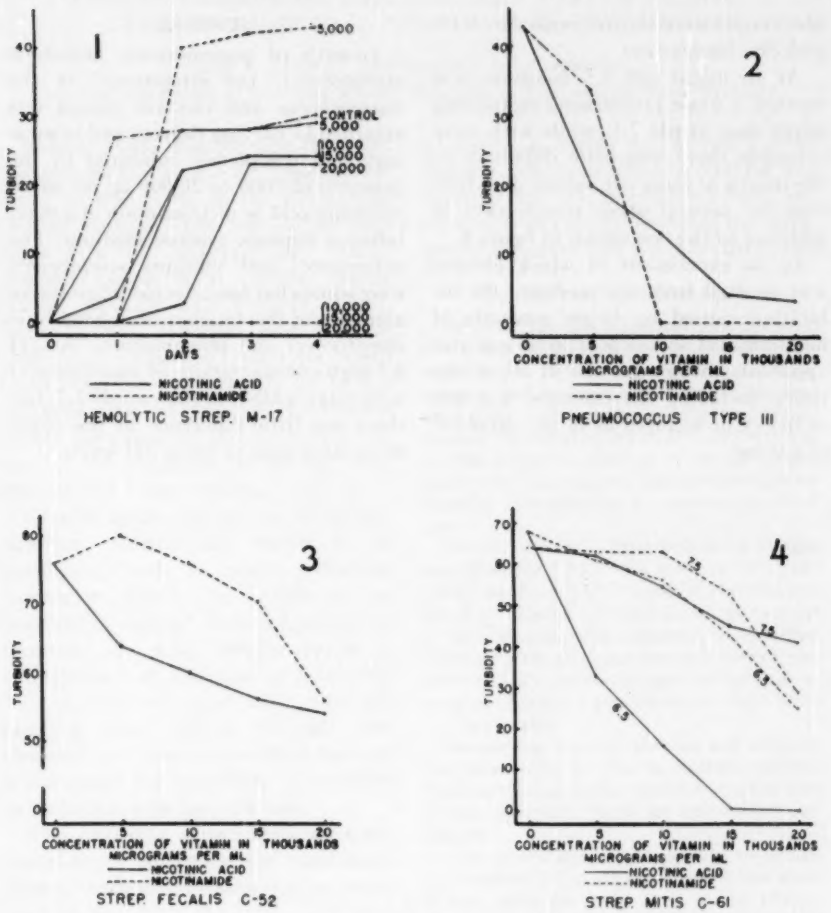

FIG. 1.—Effect of high concentrations of vitamin on initiation of growth and amount of growth. Figures at the right represent amount of vitamin in μg per ml of medium. The control tube without vitamin is shown by the dash and dot line. Initial pH of medium was 7.4.

FIG. 2.—Growth at 24 hours in different concentrations of vitamin. Results at 48 hours were essentially the same. Initial pH of medium 7.3.

FIG. 3.—Growth at 48 hours. Later readings showed little change. Initial pH of medium 7.4.

FIG. 4.—Inhibitive effects at initial pH levels of 6.5 and 7.5. Results at 72 hours.

for occasional slow growth in the presence of 10,000 μg of nicotinamide.

Several pneumococci were markedly affected by concentrations of the vitamin greater than 5000 μg per ml. Data for a type III culture are shown in figure 2. *Streptococcus fecalis* was less affected and was able to grow in the presence of 5000 to 20,000 μg per ml with relatively slight retardation in the rate of growth and the degree of turbidity finally attained (fig. 3). Compar-

able results were secured with *Str. mitis* and *Str. liquefaciens*.

At an initial pH 6.5 nicotinic acid exerted a more pronounced restraining effect than at pH 7.5, while with nicotinamide there was little difference in the results at these pH values. This held true for several other streptococci in addition to the one shown in figure 4.

In an experiment in which glucose was omitted from the medium, the inhibition caused by larger amounts of nicotinic acid and nicotinamide was also apparent, though growth in all of the tubes, including the controls, was not as heavy or as rapid as in the presence of glucose.

SUMMARY

Growth of pneumococci, hemolytic streptococci, and streptococci of the enterococcus and viridans groups was retarded in varying degrees and in some instances completely inhibited in the presence of 5000 to 20,000 μg per ml of nicotinic acid or nicotinamide in a meat infusion peptone glucose medium. The enterococci and viridans streptococci were somewhat less affected by excessive amounts of the vitamin than hemolytic streptococci and plneumococci. At pH 6.5 high concentrations of nicotinic acid were more inhibitive than at pH 7.5, but there was little difference in the effect of nicotinamide at these pH levels.

CHEMOTHERAPY OF *TRICHOMONAS FOETUS* IN THE CHICKEN EMBRYO. I. CULTIVATION TECHNIQUES

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Nelson (1938) was the first to report the successful culture of *Trichomonas foetus* in the fluids of the developing chicken embryo. The inoculation was made into the allantoic sac. Subsequently, Levine et al (1939), Hogue (1939), McNutt and Trussell (1941), Byrne (1942), Geurden and Willems (1941) and Gelormini (1942) confirmed these observations using 7- to 14-day embryonating chicken eggs. Florent (1947) has reported the successful culture of the organism in the amnionic fluid of the 7-day embryo.

Careful evaluation of the literature, however, showed the results to be conflicting, and, in many instances, lacking in detail. The number of organisms inoculated was not often determined and could not be related to the duration of infection or mortality. The number of eggs used was frequently insufficient to warrant conclusions, and previous authors had not investigated the possibility of infection by injection into the yolk sac.

The following experiments were undertaken to determine the most satisfactory method of making routine inoculations of *T. foetus* which would yield uniform infections. Such a method was essential for future chemotherapy experiments and for determining the duration of infection and the mortality rate.

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* Wellcome Research Fellow of the Animal Health Trust (from the Ministry of Agriculture and Fisheries Laboratories, Weybridge, England).

MATERIALS AND METHODS

Fertile chicken eggs which had been incubated for 10 days at 38 to 39 C were injected with a suspension of *T. foetus* into the allantoic, amnionic and yolk sac and intravenously. The eggs were sealed with colodion or adhesive cellulose tape and incubated further at 37 C until removed for microscopic examination of the egg content for *T. foetus*. Each egg was then also examined for bacterial contamination by streaking on blood agar.

The BR strain of *T. foetus* isolated by Morgan and Wisnicky (1942) was grown in Morgan's (1948) modification of Schneider's (1942) medium for 48 to 96 hours and used for the preparation of the inoculum. The organisms were washed twice in 0.85% saline and resuspended to a concentration of 1 million organisms per ml in a medium consisting of 1 part 1% tryptic digest broth to 2 parts saline.

Inoculations into the allantoic and amnionic sacs were carried out after the methods described by Beveridge and Burnet (1946), using an all glass syringe to inject 1 ml of the trichomonad suspension.

Yolk sac inoculations were made by thrusting a needle about 1.5 cm through a hole drilled about 6 mm above the lower limit of the allantochorion, avoiding any large blood vessels, and on the side opposite that occupied by the embryo. A little fluid was withdrawn to check the position of the point of the needle before the injection was made.

In order to reduce the volume of the inoculum for the intravenous inoculations, 0.2 ml of suspension containing 1 million organisms was employed. The method employed was that of Beveridge and Burnet (1946) with the exception that the eggs were sealed with adhesive cellulose tape.

PROCEDURE

Twenty-five eggs were employed in the routine experiments. Twenty of each group were inocu-

lated with *T. foetus* suspensions by a given route (allantoic, amnionic or yolk sac) and 5 served as controls to injection of 1.0 ml of saline broth. Each group of inoculated eggs was divided at random into 4 equal lots. The 1st lot was sacrificed and examined for *T. foetus* and bacterial contamination on the 2nd day after inoculation, the 2nd lot on the 4th day, the 3rd lot on the 6th day and the 4th was permitted to incubate through the normal hatching period.

The injected eggs were candled daily, and those embryos which had died before the selected day for sacrifice or before hatching were opened immediately and examined for the presence of *T. foetus*. All were cultured for the detection bacterial contaminants.

Only 10 eggs were inoculated by the intravenous method, 5 with *T. foetus* and 5 with 0.2 ml saline-broth alone. These inoculated eggs were examined on the 2nd, 3rd and 4th days, the control eggs in which the embryos succumbed being subjected to bacteriological examination.

TABLE 1.—*Inoculation of embryonating eggs via the allantoic chamber with 1 million* T. foetus.

Experimental procedure	Egg no.	Day of death (D) or sacrifice (S) after injection	*T. foetus* microscopic examination	Bacteria present
Uninterrupted incubation after inoculation	1	14D	+	−
	2	6D	+	−
	3	13D	+	−
	4	14D	+	−
	5	13D	−	−
For test at 2nd day	1	1D	+	−
	2	2S	+	−
	3	2S	+	−
	4	2S	+	−
	5	2S	+	−
For test at 4th day	1	4S	+	−
	2	4S	+	−
	3	4S	+	−
	4	4S	+	−
	5	4S	+	+
For test at 6th day	1	6S	+	−
	2	6S	+	−
	3	6S	+	−
	4	1D	+	−
	5	6S	−	−
Uninterrupted incubation (controls)	1	13D		−
	2	13D		−
	3	13D		−
	4	4D		−
	5	6D		−

RESULTS

The results of the inoculation of a *T. foetus* suspension into the allantoic, amnionic and yolk sac are recorded in tables 1, 2 and 3, respectively.

The results recorded in table 1 (allantoic sac inoculation) show a low degree of mortality before the selected day of

TABLE 2.—*Inoculation of embryonating chicken eggs via the amnionic chamber with 1 million* T. foetus.

Experimental procedure	Egg no.	Day of death (D) or sacrifice (S) after injection	*T. foetus* microscopic examination	Bacteria present
Uninterrupted incubation after inoculation	1	1D	+	−
	2	11D	+	−
	3	2D	+	−
	4	3D	+	−
	5	8D	+	−
For test at 2nd day	1	2S	+	−
	2	2S	+	−
	3	1D	+	−
	4	2S	+	−
	5	2S	+	+
For test at 4th day	1	4S	+	−
	2	1D	+	−
	3	4S	+	−
	4	1D	+	−
	5	4S	+	−
For test at 6th day	1	2D	+	−
	2	1D	+	−
	3	1D	+	−
	4	1D	+	−
	5	2D	+	+
Uninterrupted incubation (controls)	1	1D		−
	2	8D		−
	3	8D		−
	4	7D		−
	5	1D		−

TABLE 3.—*Inoculation of embryonating eggs via the yolk sac with 1 million* T. foetus.

Experimental procedure	Egg no.	Day of death (D) or sacrifice (S) after injection	*T. foetus* microscopic examination	Bacteria present
Uninterrupted incubation after inoculation	1	1D	+	−
	2	4D	+	−
	3	10D	+	−
	4	9D	+	−
	5	(H)†	−	−*
For test at 2nd day	1	2S	+	−
	2	2S	+	−
	3	2S	+	−
	4	2S	+	−
	5	2S	+	−
For test at 4th day	1	3D	+	−
	2	3D	+	−
	3	4S	+	−
	4	4S	+	−
	5	4S	+	−
For test at 6th day	1	6S	−	−
	2	6S	+	−
	3	6S	+	−
	4	6S	+	−
	5	6S	+	−
Uninterrupted incubation (controls)	1	11D		−
	2	12D		−
	3	11D		−
	4	(H)		−*
	5	(H)		−*

* Yolk sac.
† Hatched.

sacrifice, and all eggs except two, one examined on the 6th day and one dead on the 13th day, were positive for *T. foetus*. No chicks hatched in either the control group or infected group of eggs. The majority in each group survived through the time of hatching, and failure to hatch was obviously due to environmental conditions rather than trichomonad infection.

After amnionic inoculation (table 2) there was a higher initial mortality which appeared to be due to the manipulation of the embryo rather than to the inoculum. All the eggs were positive for *T. foetus* on microscopical examination, and none of the embryos hatched.

The results following inoculation into the yolk sac revealed infection at the time of examination in all but two embryos, one which hatched normally and one which was sacrificed on the 6th day (table 3).

The intravenous inoculation of 1 million trichomonads into 5 embryos gave negative results for *T. foetus* on the microscopical examination of the blood and extraembryonic fluids.

Lysis of the membranes was not observed in any of the eggs examined in these experiments nor was there any putrefactive odor.

SUMMARY

An infection with *Trichomonas foetus* (strain BR) was produced by the injection of 1 million organisms into the allantoic, amnionic and yolk chambers of embryonating 10-day chick eggs, but not by the intravenous route of inoculation. Of 60 eggs inoculated into the allantoic, amnionic and yolk chambers only 4 were negative at the time of examination, 2 on the 6th day, 1 on the 13th day and 1 which hatched. It appeared that the trichomonads per se had little influence on the development of the embryo and that the infection could persist until the time of hatching.

Inoculation via the allantoic chamber is a simple procedure, and it produced only negligible mortality of the chicken embryos. An inoculum of 1 million of strain BR *T. foetus* produced a high percentage of persistent infections of the allantoic fluid.

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CHEMOTHERAPY OF *TRICHOMONAS FOETUS* IN THE CHICKEN EMBRYO. II. DRUG SCREENING EXPERIMENTS

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The treatment of bovine trichomoniasis, particularly in the bull, has been unsatisfactory since the etiological agent, *Trichomonas foetus*, was first recognized. The nature of the infection in both the male and female is such that clinical trials with any extensive series of drugs is impractical. Other methods have been tried with limited success; in vitro tests have not proved very helpful. Morgan et al,[1] by means of an experimental vaginal infection in the rabbit, tested the trichomonacidal properties of various drugs. Certain rabbits were refractive to the infection, however, and the labor involved limited the number of drugs which could be tested. Furthermore, the possibility of spontaneous recovery during the course of drug treatment could not be entirely eliminated. Several workers have reported the successful cultivation of *T. foetus* in the fluids of the developing chicken embryo. Their work is summarized and confirmed in part I of this paper.[2] Sufficient data was also obtained upon which to base a method for testing certain compounds for trichomonacidal activity in the developing chicken embryo.

MATERIALS AND METHODS

Chicken eggs were incubated at 38 to 39 C for 10 days. Selected embryonating eggs were inoculated into the allantoic chamber with 0.5 ml of a saline-broth suspension containing 1 million motile trichomonads. The technique of the allantoic chamber inoculation and the preparation of the suspension for injection was similar to that described by Pierce and Morgan.[2]

The infection was allowed to establish itself for 24 hours. During this period any embryonic mortality immediately following the infective inoculation could be detected and eggs discarded. The drugs under test were then injected into the yolk sac, using the same technique for their administration.[2] Initially, the drugs were introduced in solutions or suspensions at a concentration of 10 mg per ml. Four eggs per lot received 1.0, 0.5, 0.3, 1.0 ml or 10, 5, 3 and 10 mg quantities of each drug, respectively.

The eggs were candled the day following the drug injection. Any dead embryos were examined for motile *T. foetus* and plated on blood agar to check for bacterial contamination. The 3rd day following the inoculation, i.e., at 48 hours following the drug injection, all the eggs were examined for motile *T. foetus* and plated on blood agar. The dead embryos were recorded and the experiment terminated.

Any drugs found to have some activity in the preliminary tests were retested over a greater dilution range and checked by further tests.

RESULTS

Table 1 lists the drugs which were found to be inactive in the primary tests against *T. foetus*. Details of the toxicity for the embryo and of bacteriological examinations which were infrequently positive are available, but as the fluids in these tests were almost 100% positive

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TABLE 1.—*Compounds inactive at a maximum concentration of 10 mg in 1 ml per egg when injected into the yolk sac of the developing chicken embryo infected with* T. foetus.

1. 3-Acetylamino-4-hydroxyphenyl-di-(carboxymethylthio) arsenite*
2. Actidione*
3. 4-aminoacetyl-aminophenyl-di-(carboxymethylthio) arsenite*
4. 9-aminoacridine (partially soluble)
5. 2-amino-4-methyl-6-dodecylamino-pyrimidine (partially soluble)
6. 4-aminosalicylic acid*
7. Antimycin "A" (suspension)
8. 2-(4-arsonophenyl)-2, 1, 3-triazole
9. Aureomycin
10. Bacitracin (maximum 800 units)
11. bis (bromophenyl) iodonium chloride (suspension)
12. bis (t-butylphenyl) iodonium chloride
13. *p*-[bis-(carboxymethylmercapto) arsino] benzene sulfonamide*
14. bis (2-hydroxyethyl) dithiocarbamate (sodium salt)
15. bis (piperidinyl-methyl) sulfide dihydrochloride
16. Bromanil (partially sol. 95% ethyl alcohol)
17. 3-buten-2-1, 4-(o-hydroxyphenyl)
18. n-butyl carbamate
19. 4-carbamidophenyl-di-(carboxymethylthio) arsenite*
20. Carbarsone oxide (suspension)
21. Cetylpyridinium chloride [ceepryn-aqueous sol. 1:1000 (isotonic)]
22. Chloral hydrate
23. Chloranil (partially sol. 95% ethyl alcohol)
24. 7-chloro-4-bis (dimethylamino-2-propylamino) quinoline
25. 7-chloro-2-*p*-chlorophenyl-4 (1′, 3′-bis-dimethylamino-2′-propylamino) quinoline trihydrochloride (suspension)
26. 5-chloro-2-mercapto benzimidazole (sol. sodium carbonate)
27. Cobalt nitrate
28. Cyanine dye derived from *n*-ethyl benzothiazole
29. *a*, *b*-dibromocinnamic acid
30. *a*, *b*-dibromopropionic acid
31. *a*, *b*-dibromosuccinic acid
32. Diethylcarbamazine (caricide)
33. Diethyl hexadecyl *b*-hydroxyethyl ammonium chloride
34. Diiodohexamethyldiamino isopropyl alcohol
35. 1, 2-dihydroxy-1-phenyl ethane phosphonic acid
36. 3, 5-diiodo-4-hydroxybenzoic acid
37. 3, 5-*l*-diiodotyrosine
38. Ethoxy-2-diamino-6, 9-acridine lactate (rivanol)
39. Ethyl alcohol (95%)
40. *b*-ethyl, γ-propyl (diisothiourea) sulfide dihydrocyloride
41. Furylacrylic acid (suspension)
42. γ-guanylmercaptopropyl-*b*′-guanylmercaptoethyl sulfide dihydrocyloride
43. Gentian violet
44. *b*-guanylmercaptoethyl *b*′-methyl *b*′-guanylmercaptoethyl sulfide dihydrochloride
45. Guinea green BA
46. Hexamethyleneisothiourea dihydrobromide
47. Iodanil (partially sol. 95% ethyl alcohol)
48. Iodine, 5 g., +potassium iodide, 10 g., + water, 100 ml (Lugol's soln.)
49. Iodoacetic acid
50. *m*-iodobenzoic acid*
51. *p*-iodobenzoic acid*
52. *b*-iodopropionic acid
53. *p*-[N(γ-mercuroxy-*b*-methoxypropyl) sulfamyl] phenylmercaptoacetic acid*
54. 2-methoxy 6-chloro-9(2′-diethylamino-4′-butylamino) acridine dihydrochloride
55. 2-methoxy 6-chloro-9-4′(diisopropylaminobutylamino) acridine dihydrochloride
56. 2-methoxy 6-chloro-9 (*a*-di-*n*-propylaminopropylamino) acridine dihydrochloride
57. *b*-methoxy-γ-mercuroxy-anhydrononylic acid*
58. Methyl blue (ink blue)
59. Myristyl γ picolineum chloride (quatresin son. 1:1000)
60. *p*-nitrobenzyliso-thiourea hydrobromide
61. 2-pentadecyl-2-imidazoline-1-ethanol (sol. dil. hydrochloric acid)
62. Pentaquine [6-methoxy 8-(5-isopropylaminopentylamino) quinoline phosphate]
63. N (1-phenyl-2-propyl) ethylenediamine (1:100 liquid)
64. Potassium dithioformate
65. Potassium iodide (20 mg. dissolved in 1 ml of physiological saline)
66. Propylene glycol diproprionate (1:100 liquid)
67. Pyridylmercuric benzoate (suspension)
68. Pyridylmercuric chloride (suspension)
69. Quinacrine hydrochloride

TABLE 1.—*Continued*

70. Silver iodide (partially soluble)
71. Sodium ethylmercurithiosalicylate (merthiolate 1:1000)
72. Sodium *n*-butylsulfonate
73. Sodium diethyldithiocarbamate
74. Sodium hydrogen sulfosalicylate
75. Sodium *a*-hydroxymercuri-*b*-methoxyhydrocinnamate (suspension)
76. Sodium 3-(γ-hydroxymercuri-*b*-methoxypropyl)-4-hydroxybenzoate
77. Sodium isopropylsulfonate
78. Sodium orthio-iodohippurate (hippuran N.N.R.)
79. Sodium phenolphthaleminsulfonate
80. Sodium ricinoleate
81. Sodium salicylate (suspension)
82. Sodium salt *p*-carbaminophenyl-arsonic acid (sodium salt of carbarsone)
83. Stannous chloride (suspension)
84. *b*-styrene phosphonic acid
85. Subtilin
86. Sulfaquinoxaline
87. Surfacaine (cyclomethylcaine)
88. 2-tridecyl-2-imidazoline-1-ethanol (sol. dil. hydrochloric acid)
89. 2, 3, 5-triiodobenzoic acid*
90. Tolu-p-quinone
91. Undecylenic acid (1:100 solution)
92. 2-undecyl-imidazole (partially soluble)
93. 2-undecyl-2-imidazoline (sol. dil. hydrochloric acid)
94. Uranium acetate (suspension)
95. Urea
96. Victoria green S (malachite green, extra concentration)
97. *b*-vinyl naphthalene-*b*′-phosphonic acid
98. p-xyleneisothiourea dihydrobromide
99. Zinc difluoroethyl dithiocarbamate (partially soluble)

* Rendered soluble by the addition of an equal weight of sodium bicarbonate.

TABLE 2.—*Compounds showing activity against* T. foetus.

Drug	Egg no.	Death* or sacrifice	Bacterial culture	*T. foetus* microscopic examination	Mg per egg
p-[bis-(carboxymethylmercapto) arsino] acetanilide	1	D2	–	–	20
	2	D2	–	–	20
	3	D2	–	–	15
	4	D2	–	–	10
	5	D2	–	–	5
	6	D2	–	+	3
	7	S3	–	+	2
	8	S3	–	+	1
	9	D2	–	+	2
p-[bis-(carboxymethylmercapto) arsino] benzamide	1	D2	–	–	20
	2	D2	–	–	20
	3	D2	–	–	15
	4	D2	–	–	10
	5	D2	–	–	5
	6	S3	–	–	3
	7	S3	– (few)	+	2
	8	S3	–	+	1
	9	S3	–	+	2
4-carbamidophenyl-di-(1′-sulfonic acid-4′-thiophenyl) arsenite	1	D2	–	–	20
	2	D2	–	–	20
	3	D2	–	–	15
	4	D2	+	–	10
	5	D2	–	+	5
	6	D2	–	+	3
	7	D2	+	+	2
	8	D2	–	+	1
	9	S3	–	+	2
Uninterrupted incubation (controls)‡	10	Hatched	–†		

All compounds were rendered soluble by the addition of an equal weight of sodium bicarbonate.

* Large amounts of the drug consistently produced death denoting some drug toxicity.

† Yolk sac.

‡ Other controls using killed embryos showed no effect on the survival of trichomonads for 5 days.

for *T. foetus*, the data have not been included.

The data in table 1 show that a large number of drugs failed to influence the trichomonas infection of the chicken embryo at the concentration specified and under the conditions of the test. Many of these drugs probably would not have been effective in vitro, and their elimination by screening with the chicken embryo method represents an economical procedure.

The activity shown by the three drugs included in table 2 would warrant further study of them, particularly, *p*-[Bis-(carboxymethylmercapto) arsino] benzamide, which had been reported as being highly effective and of relatively low toxicity in the treatment of filariasis in man.

SUMMARY

An in vivo technique utilizing the embryonating chicken egg has been described for determining possible trichomonacidal properties of various compounds against *Trichomonas foetus*. A total of 102 compounds was tested by this method, of which the following three showed some activity: *p*-[Bis-(carboxymethylmercapto) arsino] acetanilide, *p*-[Bis-(carboxymethylmercapto) arsino] benzamide and 4-carbamidophenyl-di-(1′-sulfinic acid-4′-thiophenyl) arsenite.